Autoantibodies, genetic polymorphisms and clinical subsets in systemic sclerosis

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AUTOANTIBODIES, GENETIC POLYMORPHISMS AND CLINICAL SUBSETS IN SYSTEMIC SCLEROSIS

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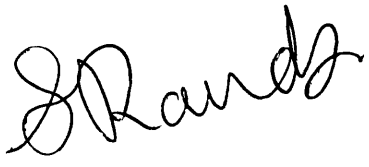

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To my late brother

Martin

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ABSTRACT

Systemic sclerosis is an autoimmune disease characterised by fibrosis and serological autoantibodies (Aabs), some of which are indicative of disease subtype. A degree of Aab mutual exclusivity gives rise to three, major serological groups. Genetic associations with SSc become stronger when autoantibody subgroups are considered. There are ethnic differences in the pattern of disease and autoantibody frequencies.

The aims of this thesis are to further understanding of the aetio-pathogenesis of SSc by; investigating autoantibody specificity, identifying possible genetic predetermination or restriction of Aab generation and exploring the relationship of these two factors with disease profile, course and outcome. A variety of standard serological and genetic methods have been used in this investigation.

Several important findings have been made in the present study: I) Although patients with anti-topoisomerase I antibodies (ATA) or anti-RNA polymerase III antibodies (ARA III) represented distinct groups, their mutual exclusivity could not be explained by the genetic analysis in this study: II) ATA positive SSc patients and normal controls showed T cell proliferative responses to recombinant topoisomerase I, and shared a common genetic background. This is consistent with the notion that auto-reactive T cells are part of the immune system repertoire and the ability to mount a T cell proliferative response is genetically restricted: III) HLA background, autoantibody profile and clinical course are ethnically variable, however associations between these three factors are independent of ethnicity: IV) The majority (91%) of ACA positive patients had lcSSc and an association with HLA DR1 and DR4: V) Consideration of the genetic contribution to fibrosis identified microsatellite marker associations with TGFβ2 and TGFβ3 indicating that polymorphisms exist in these genes that predispose individuals to SSc. Stratification according to serological groups showed similar associations with microsatellite marker as stratification according to skin type.

Together these results provide evidence to suggest a genetic susceptibility to specific autoimmune responses in SSc. Existing HLA associations are supported and new ones are proposed. Polymorphisms of specific fibrosis genes may exist that are associated with disease. Overall, the relationship between HLA-autoantibody-disease subset has been supported and defined to some degree. Increased understanding of this relationship may further elucidate the pathogenic mechanism underlying SSc and ultimately improve the diagnostic and prognostic value of autoantibodies as disease markers.

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ABBREVIATIONS

Ab	Antibody
Aab	Autoantibody
ACA	Anticentromere antibody
ACE	Angiotensin-converting enzyme
AMP	Ammonium persulphate
ANA	Anti-nuclear antibody
ANoA	Anti-nucleolar antibody
APC	Antigen presenting cell
ARA	Anti-RNA polymerase
ATA	Anti-topoisomerase I antibody
BC	British Caucasian
BCIP	5-bromo-4-chloro-3-indolyl phosphate
B. corr.	Bonforroni correction
Bp	Base pairs
CENP	Centromere Protein
CTD	Connective tissue disease
CTE	Calf thymus extract
DcSSc	Diffuse cutaneous systemic sclerosis
DdH_2O	Double distilled filter purified water
DFCS	Dialysed foetal calf serum
DsDNA	Double stranded DNA
DNA	Deoxyribose nucleic acid
EC	Endothelial cell
FCS	Foetal calf serum
FITC	Fluorocein isothiocyanate
G	Gram
GVHD	Graft versus host disease
HLA	Human leukocyte antigen
IB	Immunoblotting
ICAM	Intercellular adhesion molecule
IDDM	Insulin dependent diabetes mellitus
IF	Immunofluorescence

IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IP	Immunoprecipitation
IPP	Protein immunoprecipitation buffer
LcSSc	Limited cutaneous systemic sclerosis
L	Litre
MCTD	Mixed connective tissue disease
MHC	Major histocompatibility complex
Nm	Nanometer
NK Cell	Natural killer cell
OD	Optical density
PAGE	Polyacrylamide agarose gel electrophoresis
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PDGF	Platelet derived growth factor
Pmol	Picomol
PF	Pulmonary fibrosis
PVC	Polyvinyl chloride
RA	Rheumatoid arthritis
RNHRD	Royal National Hospital for Rheumatic Diseases
ROS	Reactive oxygen species
RP	Raynauds's phenomenon
RPEs	Relative predispositional effects
RPMI	Rosewell Park Memorial Institute medium
rRNP	Ribosomal RNP
RTE	Rabbit thymus extract
SD	Scleroderma-like disease
SDS	Sodium dodecyl sulphate (AKA Sodium laureth sulphate)
Sec	Second
SI	Stimulation index
SLE	Systemic lupus erythematosus
SAB	South African Black
SNP	Single nucleotide polymorphism

SS	Sjogren's syndrome
SSc	Systemic sclerosis
TBE	Tris borate EDTA
Tc Cell	Cytotoxic T lymphocyte
TCR	T cell receptor
TEMED	N,N,N',N'-tetramethylethylenediamine
TGF	Transforming growth factor
Th Cell	Helper T lymphocyte
TNF	Tumour necrosis factor
Topo-I	Topoisomerase I
Tris.Cl	Tris[hydroxymethyl]aminomethane hydrochloride
Trizma base	Tris[hydroxymethyl]aminomethane hydrochloride
Ts Cell	Suppressor T lymphocyte
Tsk	Tight skin mouse
UCTD	Undifferentiated connective tissue disease

CHAPTER 1

INTRODUCTION

1.1: Clinical features of systemic sclerosis

Systemic sclerosis (SSc) is a heterogeneous, connective tissue disease, characterised by fibrosis, a spectrum of degenerative changes involving the blood vessels, skin, and certain internal organs, and the presence of disease-specific serological autoantibodies. The hallmark of the disease is thickening of the skin (scleroderma) caused by increased deposition of extracellular matrix and excessive accumulation of connective tissue.

Disease profile and skin involvement

Raynaud's phenomenon is usually the first symptom of SSc, and affects the vast majority of patients (1). Episodes are a result of intense vasospasm of peripheral arteries, which may be triggered on exposure to cold or emotion. The fingers (and less commonly the toes) become initially very pale and then blue from cyanosis secondary to the poor blood flow. Eventually when the blood flow returns there is redness and discomfort. Although Raynaud's phenomenon is prevalent in some 30% of normal women only a small percentage of individuals go on to develop SSc (1).

Systemic sclerosis can be divided into 2 major variants: *diffuse cutaneous* (dcSSc) and *limited cutaneous* scleroderma (lcSSc), dependent primarily on the degree and extent of cutaneous involvement (2). Three phases can be recognised in the development of the disease. During the *edematous phase* there is often oedema ('sausage' swelling) and restriction of movement of the fingers, which can extend to involve the forearms, legs, feet and face. This may then be replaced gradually by thickening and tightening of the skin (*indurative phase*) over the course of several weeks or months. In the majority of patients (60%) these changes tend to be restricted to the fingers, hands and face in lcSSc, but spread at a variable rate to the forearms, upper-arms, thighs, chest and abdomen in the diffuse form of the disease. The skin becomes increasingly shiny and taut, impairing the mobility of the joints and face. In limited disease, skin thickening is much less prominent and the most striking finding maybe numerous telangiectasic lesions on the digits and face. Telangiectatic lesions are recognised as red spots, which blanche on pressure, resulting from a localized collection of distended blood capillary vessels. After several years, during the final *atrophic phase* the affected skin tends to soften and return to a normal thickness or may become thinner than normal.

The onset of lcSSc usually follows a long duration of Raynaud's and the skin thickening tends to remain minimal over many years. In contrast dcSSc, often shows an early, accelerated, increase in skin thickness following a short duration of Raynaud's.

Further clinical manifestations

Fibrotic damage often occurs in internal organs in addition to the skin. Rapid progression in skin thickness is associated with several internal organ problems.

Pulmonary Involvement

Pulmonary disease has become the major cause of death in SSc patients (3). Pulmonary hypertension (PH) and pulmonary interstitial fibrosis (PF) are the two major pulmonary conditions occurring among SSc patients. Pulmonary disease is evaluated most often by pulmonary function test and chest radiograph (1). High resolution CT scanning and echocardiography are useful procedures for further investigating the possibility of PH and PF respectively. Severe PH is clinically detectable in nine percent of lcSSc patients where the prevailing symptom is dyspnoea and the most common physiologic abnormality is a reduced diffusing capacity. PH is rare in the dcSSc variant but may present secondary to interstitial lung disease. PF may be present in both variants of the disease; occurring in up to 70% of dcSSc patients, but is comparatively rare in lcSSc patients. Again, the major clinical feature is dyspnoea, which may be accompanied by a cough (4). Pulmonary function tests reveal reduced lung capacities and chest radiographs may show linear and reticular shadows (for review see Silver '90) (5).

Renal Involvement

Renal disease occurs in approximately ten percent of all scleroderma patients (6), and was the major cause of SSc patient death until the introduction of angiotensin converting enzyme, (ACE), inhibiting drugs. Scleroderma renal crisis typically occurs early in those patients with rapid, progressive skin thickening (dcSSc) (~20%) (6;7). Early indicators of renal involvement include proteinuria, hypertension and impaired glomerular filtration. A vascular aetiology is reflected in the histopathologic changes, which reveal intimal thickening of the small and medium arteries accompanied by medial atrophy and luminal occlusion (4). Fibrosis and necrosis may also be seen in later stages of advanced disease.

Gastrointestinal tract involvement

Involvement of the gastrointestinal (GI) tract organs is apparent in approximately 90% of patients. Of these patients, 90% will probably suffer oesophageal disease, although any part of the tract may be involved (8). Pathologic changes consist of fibrosis, atrophy and distinctive changes in vasculature with consequent problems such as malabsorption, dysphagia, heartburn and vomiting.

Cardiac involvement

Myocardial fibrosis is the hallmark of cardiac involvement in SSc. The fibrosis is patchy and distributed throughout the myocardium. Cardiac involvement is suggested to be prevalent in approximately 20% of patients and is seen in both subsets of the disease.

Other clinical manifestations

Skeletal muscle involvement is common in SSc (1). There is mild weakness and atrophy of the muscles with a minimal elevation of creatine phosphokinase. A small number of patients suffer inflammatory myositis, indistinguishable from polymyositis (7;9). Neurologic involvement is rare (1), however reports describe disease affecting the autonomic, peripheral and central nervous systems, which is thought to arise via a diversity of pathogenetic mechanisms (10).

SSc variants and overlap syndromes

'Localised scleroderma', and 'Systemic sclerosis sine scleroderma' are two further variants of the disease. The former is characterised clinically as isolated patches of inflamed, fibrotic skin (11). SSc sine scleroderma is a rare variant, which does not involve the skin (12). (These two variants will not be considered in this research).

In addition to having SSc, some patients also exhibit features of other CTDs. Such cases are termed overlap syndromes. Patients with overlap present with a combination of major features of more than one disease: clinically and often serologically (13). The most common overlap syndromes are mixed connective tissue disease (MCTD), scleromyositis and synthetase syndrome (14). Systemic lupus erythematosis (SLE), Sjogren's syndrome (SS) and rheumatoid arthritis may also be seen in overlap with SSc.

1.2: Classification of SSc

Scoring and classification systems

Several scoring and classification systems have evolved in the evaluation of scleroderma. A recent contribution by Medsger and colleagues employed a scoring system to assess the involvement of nine organ systems to assess disease severity (15). The most widely accepted classification of SSc delineates two subsets of the disease (dcSSc and lcSSc) (2), and is used to define SSc patient subsets in the present work. Using this system, the primary determinant of patient classification is the extent of cutaneous involvement. Visceral and serological manifestations are also considered, as shown in table 1.1:

Table 1.1: Classification of SSc disease subsets

dcSSc	lcSSc
Truncal and acral skin involvement Presence of tendon friction rubs	Skin involvement limited to hands, face, feet, and forearms (acral) or absent
Onset of Raynaud's within 1 year of onset of skin changes	Raynaud's for years (occasionally decades)
Early and significant incidence of interstitial lung disease, oliguric renal failure, diffuse gastrointestinal disease, and myocardial involvement	A significant late incidence of pulmonary hypertension, with or without interstitial lung disease, trigeminal neuralgia, skin calcifications, telangiectasia
Nailfold capillary dilation and capillary destruction	Dilated nailfold capillary loops, usually without capillary dropout
Anti-topoisomerase-I antibodies (30%) of patients Absence of anticentromere antibodies	A high incidence of ACA (70-80%)

From LeRoy et al. 1988 (2)

A system (originally) described by Barnett *et al.* ('78) classifies SSc disease further. This system subdivides lcSSc into two parts: type I includes patients with sclerodactyly and type II those patients with skin changes beyond the fingers but remaining in the extremities. Type III includes dcSSc patients (16;17).

Autoantibody status

SSc is classed as an autoimmune disease. More than 90% of patients have autoantibodies present in their serum. Certain autoantibodies are disease specific and characteristic of disease subset, and thus aid diagnosis and prognosis. As they are strongly implicated in the aetiology of SSc, they are discussed in section 1.5.

1.3: SSc Epidemiology

Prevalence and incidence

The prevalence of SSc has been demonstrably stable since 1973, and is reported to affect 100-200 persons/million. The onset of disease is typically seen between the ages of 30-50, although can occur at any age (18). Overall, women are affected 4 times as often as are men (20; 22), and this sex difference is increased during the child bearing years. The disease is considered rare with an annual incidence of under 10/million (19). However, incidence rates from a number of countries vary considerably, ranging between one and 20/million population/year (18).

Mortality, survival and prognosis

The overall, annual, average population mortality from SSc is approximately 1 to 3/million (18). In obtaining reliable survival statistics many methodological issues, encompassing patient recruitment, referral delay and loss of patients to follow-up, are encountered (18). However, with these factors considered, median survival figures of approximately 60% at five years and 50% at ten years are suggested (18). Risk factors for reduced survival include the presence of diffuse disease, older age of onset and major organ involvement (20). Advances in therapeutic intervention have led to some decrease in mortality.

Race and ethnicity

There is evidence to suggest that racial and ethnic background influence disease susceptibility, clinical expression, and serologic markers characteristic of the disease (reviewed by Mayes '96) (20). There is a higher incidence in Blacks compared to Whites (20-22). Black females are more likely to be diagnosed at a younger age and have more aggressive disease (21;22). Prevalence of specific serologic markers also

varies with ethnic diversity, where perhaps the most frequently reported difference is the relative lack of ACA in Black populations (21;23;24). The precise contribution of genetic background and environmental factors toward these observed differences remain to be elucidated.

Genetic factors and geographical clustering

Numerous studies have investigated the association between HLA types and SSc, and associations appear stronger when clinical sub-populations and/or serologic groups are considered (25;26). Immunogenetic associations continue to emerge and extend beyond the HLA region to encompass T-cell receptor genes and a variety of candidate genes, which will be considered later.

The prevalence of SSc varies by geographic region and is higher in the United States than in Europe or Japan (27). There have also been several reports of clustering of SSc, including areas of Britain (28;29). The most well known cluster of SSc involves the Native American Oklahoma Choctaw: a population with a conserved gene pool (30). The Oklahoma Choctaw demonstrate a high incidence of SSc, often with anti-topoisomerase I antibodies (31). Studies have revealed a genetic haplotype conferring susceptibility to SSc and an environmental trigger has remained elusive (31). There is convincing evidence that certain environmental agents are involved in the development of at least some clinical variants of SSc and will be considered later.

1.4: Pathogenesis of SSc

The precise pathogenesis of SSc is unknown. It is likely that a number of characteristic vascular, immune and fibrotic events, combine in a complex manner, and result in a self-perpetuating cycle. The general cascade of events will be discussed presently, with more detailed consideration of autoimmune and genetic factors subsequently.

Endothelial activation

As early as 1975 it was proposed that endothelial cell damage and dysfunction were the primary cause of scleroderma (32); a phenomenon considered central to the pathophysiology of SSc, and the focus of intense research today. Although the precise

mechanism by which endothelial involvement is initiated is not known, possible inciting factors of activation include Raynaud's Phenomenon, (present in the majority of SSc patients), retroviral and environmental contributors, which are discussed in section 1.6.2). Characteristics of the consequent endothelial activation and injury include loss of vascular integrity and increased permeability, expression of leucocyte adhesion molecules (ICAM-1 and VCAM-1), cytokine production, and increased surface expression of MHC class II molecules (33). An elevated plasma level of Von Willibrand factor VIII, sequestered by EC granules, is further evidence of EC activation (34).

Immune system activation

Mediators released by the endothelium cause immune activation, (which may also be brought about independently via an environmental trigger) (35). In early SSc lesions there is an inflammatory cell infiltrate which is dominated by T-cells. Subset analyses of T-lymphocyte sub-populations, in the skin, have revealed an increase in the proportion of the CD4+ (T helper) cells (36). MHC class II expression (35;37) evidences the activated state of the T-cells. Further evidence of immune activation is the raised level of the cytokine IL-2. Also the presence of autoantibodies (discussed later) implies B-cell activation. Activated lymphocytes are receptive to the adhesion molecules present on the activated endothelium and adherence contributes to vascular injury (38). Granzyme 1, which is found in SSc skin, is secreted by T-lymphocytes and is a factor thought to be cytotoxic to the endothelium (35).

Other cells also infiltrate the tissues and include natural killer (NK) cells, mast cells and macrophages, monocytes, eosinophils and basophils. Natural killer cells can be converted to lymphokine-activated killer cells, by circulating IL-2, which maybe an additional means of endothelial injury. Histamine, a factor raised in endothelial cell capillaries in SSc, indicates the accumulation of mast cells. Soluble mediators secreted by the T-cells, B-cells, and non-specific inflammatory cells can not only activate and damage endothelial and vascular cells, but also up-regulate fibroblast activity (37). The activation of immune cells and vascular degeneration precede cutaneous fibrosis.

Fibrosis

The principle cell type involved in the production of collagen and other components of the extracellular matrix is the fibroblast and it normally does so as part of a highly regulated process. Progressive fibrosis due to excessive collagen deposition is the classic hallmark of SSc. An abnormal fibroblast phenotype is recognised in scleroderma, which produces increased amounts of collagen (collagen type I, III, V, VI and VII, glycosaminoglycans, fibronectin, laminin, decorin, and tenasin) (4;39). Local factors are thought to be responsible for the activated state rather than a fibroblast defect since fibroblasts taken from unaffected skin of SSc patients display normal levels of matrix production (40) and the activated phenotype is eventually lost when the cells are cultured *in vitro* (despite displaying activity for several passages) (4).

Accumulation of inflammatory cells and various adhesion molecules, and expression or secretion of integrins is a consequence of endothelial injury and the inflammatory response. In turn this promotes the synthesis and release of various cytokines and growth factors. Two important cytokines that may contribute to fibrosis are transforming growth factor beta (TGFβ) and platelet derived growth factor (PDGF). TGFβ and PDGF mediate the proliferation and activation of vascular and other connective tissue cells, particularly the fibroblast (41). TGFβ is an indirect mitogen for fibroblasts which acts via (PDGF)-a receptor interactions (41), and an over production of TGFβ by cells in the immune infiltrate has been demonstrated (42). TGFβ causes persistent activation of the fibroblast to produce collagen by stimulating ECM components and inhibiting matrix-degrading metalloproteinases. The infiltration of platelets into the inflammatory site results in raised levels of PDGF in the locality. PDGF is chemotactic to fibroblasts and promotes their proliferation and activation. Fibroblast proliferation and collagen production is also induced by endothelin-1, a factor that is raised in SSc lesions and produced by the activated endothelium (35). Thus the vascular injury described does not only cause activation (an autocrine pathway), but also gives rise to fibroblast proliferation (35).

IFNγ is an inhibitor of collagen production. It has been suggested that the reduced secretion of this cytokine contributes to increased collagen synthesis, in a negative fashion (35). The fibrogenic cytokines IL-4 and TNF, released by activated T-cells, cause fibroblast proliferation and matrix gene expression. The activated fibroblasts may

themselves then release cytokine and growth factors with the potential of exerting paracrine or autocrine effects (41).

Breaking tolerance and autoimmunity

The elimination of autoreactive T cells in the thymus during development (negative selection) is considered the principle mechanism by which T cell autoreactivity is controlled. The negative selection of T-cells requires a sufficiently high concentration of class II bound peptide and T-cell receptor affinity. Thus, if a self-peptide is not presented, or presented in low amounts then T-cells specific for this peptide will escape deletion. The immune system of a healthy person does contain some autoreactive T and B cells. If these remaining autoreactive lymphocytes escape control mechanisms and become activated then autoimmunity will ensue.

A hypothesis regarding the mechanism by which autoreactive T cells become activated has been proposed by Casciola-Rosen *et al.* (43). They suggested that the break in tolerance seen in SSc was due to the unique fragmentation of autoantigen, which results in subsequent exposure of cryptic epitopes. It was proposed that reactive oxygen species, produced during ischaemic-reperfusion injury, in the presence of specific metal ions, which are known to accumulate inside the nucleoli of cells (Cu, Fe), might initiate altered cleavage of SSc specific antigens, giving rise to the immunogenic cryptic epitopes (43). SSc-specific autoantigens, under certain physiological conditions, are localised within the nucleolus. (Cellular localisation of antigen, metal ion concentration and the availability of metal binding sites on the antigen have been proposed as reasons for the observed autoantibody profiles seen in SSc (44)).

Once tolerance has been broken (i.e. the autoreactive T-cell has been activated by minor antigenic determinants) subsequent autoreactive B-cell activation will follow. This includes B-cell function as an APC, and can lead to the spreading of T cell responses to different epitopes of the antigen (45). In an investigation of the mechanisms controlling the antitopoisomerase I response in SSc, Kuwana *et al.* showed that T and B cell collaboration was essential (46). Further, they identified autoreactive T-cells to topoisomerase I in normal healthy controls, indicating that such cells are components of the normal immune system repertoire. Their findings also support the hypothesis that the autoimmune response in SSc is antigen-driven (46).

SSc associated autoantibodies.

Autoantibodies are present in the sera of more than 97% of SSc patients. They display associations with disease, and genetic correlations with autoantibody specificity are also emerging. Autoantibodies may be detected prior to disease onset, which suggests that they are associated with a disease-causing event (47). Possible mechanisms, (inciting events), giving rise to autoantibody production will be considered in section 1.6. While autoantibodies may not be directly pathogenic to SSc, they are certainly indicators of pathogenesis and useful in the diagnosis and prognosis of disease, and thus are a valuable area of investigation.

Anti-endothelial cell antibodies

Anti-endothelial cell antibodies (AECA) have been described in SSc patients and they represent a heterogeneous family of IgG class autoantibodies (48;49). Although their possible pathogenicity remains somewhat controversial there is mounting evidence to suggest that they play a cytotoxic role (48). It has been reported that the binding of AECA to EC induces activation of the EC and upregulation of cell adhesion molecules, possibly due to autocrine action by IL-1 (49). Also, EC programmed cell death is emerging as a central participant in the pathophisiology of vascular injury (50;51). Research in this area by Sgonc ('99) has involved the animal model of SSc, the UCD-200 chicken, whose disease includes the vascular manifestations, which is lacking in other animal models such as the tight skin mouse. A parallel comparative study of skin biopsies of UCD-200 chickens and human SSc patients revealed that EC apoptosis, is induced by AECA-dependent cellular cytotoxicity, and is a primary event in the pathogenesis of SSc (52).

1.5: Autoantibodies and autoimmune disease

Autoantibodies directed to specific intracellular antigens are a characteristic of most connective tissue diseases. In SSc, distinct profiles of autoantibodies are evident. Autoantibodies reactive toward antinuclear antigens (ANA) may be present throughout the spectrum of connective tissue diseases, or may indeed be specific for SSc only. The particular autoantibody present is often indicative of clinical expression and disease course and is thus an important aid in establishing a diagnosis and predicting prognosis.

Autoantibodies are becoming increasingly valuable for discriminating subgroups of patients that differ in prognosis or response to therapy.

Methods for identifying ANA

Ouchterlony double immunodiffusion and indirect immunofluorescence are two original methods, still used as standard, for detecting autoantibodies, particularly for clinical purposes. More recent developments have given rise to more sophisticated tests such as immunoblotting and immunoprecipitation. All of these techniques have been used in this research and are detailed in chapter 2.

SSc Autoantibodies

Three major autoantibody groups are recognized in SSc and they tend to be mutually exclusive of each other. Autoantibodies comprising each group tend to be associated with certain subtypes of the disease and may be present with other autoantibodies considered to be less disease specific.

Anti-topoisomerase I antibodies

A nuclear antigen uniquely recognized by SSc sera is the 100kDa enzyme topoisomerse I (topo-I). Topo I is localized in the nucleolus and nucleoplasm of the cell where it is involved in DNA replication and transcription, and the regulation of chromosome structure by relaxing supercoiled DNA (53;54). Anti-topoisomerase I antibodies (ATA), previously called anti-Scl-70, are present in approximately 22% of SSc patients and identify a subgroup of patients with diffuse disease and an increased risk of PF (45). Many ATA sera also contain autoantibodies recognising subunits of the enzyme RNA polymerase II, namely ARA II^A (the larger phosphorylated subunit), and ARA II^O (the non-phosphorylated subunit). It should be noted that while some sera only recognise one subunit, some recognise both (45).

Anticentromere antibodies

Autoantibodies reactive with the centromere region of cells were first described in 1980 (55). It has emerged since that a number of centromeric proteins are targeted in SSc. There are three main centromere antigens recognized in SSc; CENP-A (19kd), CENP-B (80kd) and CENP-C (140kd) (56), and their location and predicted function are shown

in table 1.2. Three further CENP proteins; CENP-D, CENP-E and CENP F, are less often recognized by autoimmune sera (57).

Table 1.2: The Major centromere autoantigens

CENP Autoantigen	Cellular location	Protein recognized and predicted function
CENP-A	Nucleosomal	Centromere specific histone H3 variant. Suggested role in the packaging of centromeric DNA.
CENP-B	Centromeric hetrochromatin, beneath the kinetochore plate	A DNA-binding protein that binds to a specific 17 base pair sequence in human alpha satellite DNA – likely to play a role in the formation of a specified structure or function of human centromere
CENP-C	Inner kinetochore plate	An essential component of a functional human centromere. Stabililization of the kinetochore.

Information from Okano '96 (53), and McHugh '96 (57)

Anti-CENP antibodies are detected in the sera of approximately 26% of SSc patients (45), who are most likely to suffer the limited form of the disease, and often show prominent vascular features (58-60). Renal manifestations are rare in ACA positive SSc patients.

Anti-RNA Polymerase III antibodies

The third major group of autoantibodies in SSc are those directed toward RNA polymerase III. RNA polymerases catalyse the transcription of different sets of genes in the cell. RNA polymerase III synthesizes small RNA, including 5s ribosomal RNA and transfer RNA, in the nucleoplasm (53). Approximately 18% of SSc patients have anti-RNA polymerase III autoantibodies (ARA III) and show the greatest risk of developing dcSSc and renal involvement (45). ARA III usually occurs together with autoantibodies recognising ARA I: an enzyme that synthesizes ribosomal precursors in the nucleolus. Further more, sera containing ARA III and ARA I often also contain autoantibodies that recognise ARA II^A and ARA II^O (45;53).

Minor antinucleolar antibodies in SSc

Other antinucleolar antibodies (ANoA) are also present in SSc that identify subsets of the disease, particularly those that overlap with other autoimmune conditions. Anti-Pm-Scl antibodies, seen in approximately four percent of patients, are often identified in those SSc patients with polymyositis overlap (61). The precise role of this autoantigen is not known but is suggested to be involved in pre-ribosomal formation (53). Anti-U3RNP antibodies are evident in some seven percent of SSc patients and are associated with an early onset of disease that usually progresses to dcSSc. (45). U3RNP is a protein involved in ribosomal RNA processing. Anti-Th RNP antibodies are occasionally identified in SSc patients (<4%), and are indicative of lcSSc and small bowel involvement (62).

Minor antinuclear antibodies in SSc

Less specific autoantibodies, directed toward nuclear proteins, are also often present in SSc sera, and include the following: Anti-Ro antibodies have been associated with severe disease that shows very rapid progression, and SSc patients with this autoantibody often have renal and pulmonary involvement. Anti-Ro antibodies are frequently seen concurring with other autoantibodies in SSc, including ATA. Antibodies reactive toward U1RNP are identified in approximately six percent of patients, and are associated with isolated pulmonary arterial hypertension, early disease onset and in patients with SLE/SSc overlap syndrome. Anti-Jo-1 (anti-histidyl-tRNA synthetase) antibodies are identified in less than five percent of patients, often those with SSc/polymyositis overlap syndrome.

Mutual exclusivity and concurrence of autoantibodies in SSc

The three major autoantibody groups ATA, ACA and ARA III are, almost without exception, regarded as mutually exclusive. As immunological techniques have become more sensitive, detection of these autoantibodies has become more reliable and, led to the recognition of other 'minor' autoantibody specificities now also considered characteristic of the disease. Analogous with this, autoantibody associations with clinical profile have also become more detailed and particular associations are now firmly recognised.

Understanding the mechanism, giving rise to the observed autoantibody mutual exclusivity, is likely to provide valuable insight into the pathogenesis of the disease. In 1988 it was suggested by Steen *et al.* that mutual exclusivity of serologic subgroups of SSc represent aetiologically distinct disease processes, or, may reflect the differences in a patients' vulnerability (63). This concept has provoked much interest and in 1998 it was proposed that SSc might actually represent a collection of separate diseases each having distinct pathogenetic origins that determine disease expression, or, may be that different autoantibody groups emerge from a common event, which is channelled via the influence of genetic background (64).

Thus, the answer to the mechanism underlying the pattern of autoantibodies recognised in SSc, the occurrence of distinct clinical subgroups, and the apparent associations between the two, may be held in an underlying genetic predeterminant. It is this concept that forms the basis of the research detailed in this report.

1.6: The aetiology of systemic sclerosis

The precise aetiology of SSc is unknown, however a range of genetic, and environmental factors, have been reported to be, and are regarded as aetiological risk factors and triggers for the autoimmune response. Studies have demonstrated that autoimmune disease is more prevalent in people with a certain genetic background. Tissue damage and the subsequent release of self-antigens are suggested to arise from exposure to environmental agents, which thus predispose an individual to autoimmune disease.

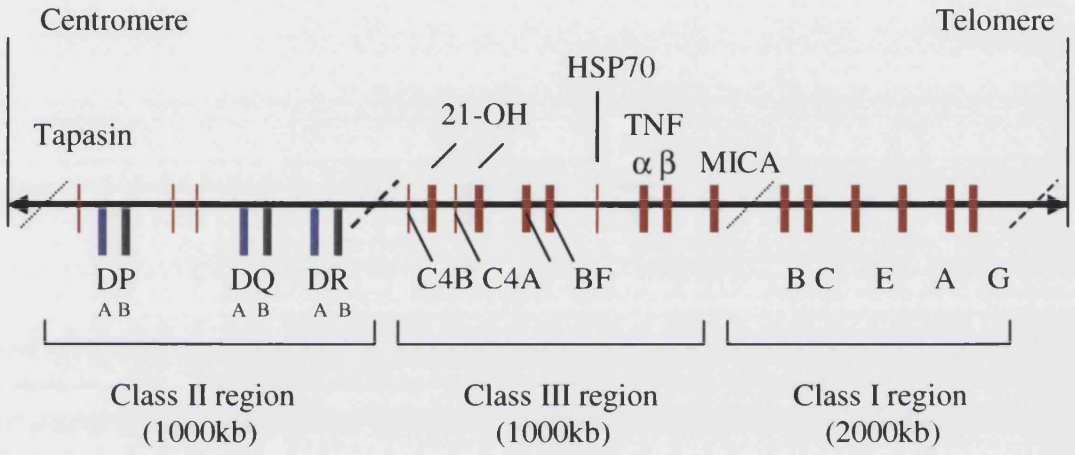
1.6.1: Genetically related factors

The role of the MHC

The HLA molecules are encoded by a cluster of genes linked on the short arm of chromosome 6 in a region known as the Major Histocompatibility Complex (MHC). These molecules form receptors, which display peptide fragments of antigen, (antigen presentation), on the cell surface where they can be recognised by appropriate T-cells.

In presenting foreign antigen to T-cells, HLA molecules evoke cytotoxic T lymphocyte and helper T cell responses, which then regulate specific immunity. The class II molecules include HLA-DR, DQ and DP, each of which is encoded by distinct genetic loci clustered in the class II region spanning approximately one megabase, as shown in figure 1.1

Figure 1.1: Schematic representation of the MHC class II complex.



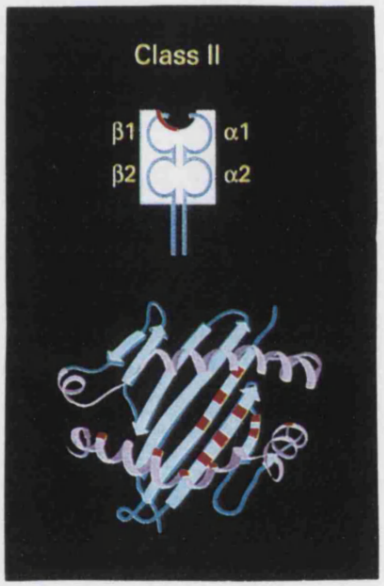
A representation of the human major histocompatibility complex (MHC), located on the short arm of chromosome 6, showing the approximate positions of distinct genes. The class II genes are located most centromerically. TAP: transporter of antigenic peptides; 21-OH: steroid 21-hydroxylase enzyme; C4A and C4B complement loci; Bf: Properdin factor B of the alternate complement pathway; C2: complement C2; TNF: tumour necrosis factor; MICA: MHC class I chain-related gene.

Structure of the MHC class II molecules

MHC class II molecules are highly polymorphic membrane glycoproteins, the structure of which is achieved by the association of two membrane bound chains; α and β (65). Together the chains form the antigen binding-cleft. The MHC class II chains are each encoded by distinct loci, which are closely linked as pairs of α and β genes. The

polymorphisms of HLA DR, DQ and DP are confined largely to the antigen-binding groove of the functional HLA molecules, but in HLA DR, they are confined to the DR beta chain (DRB gene) with the DR alpha chain (DRA gene) being essentially monomorphic (66). HLA DQ, and DP, also contain polymorphisms but in both the beta and alpha chain genes. The most polymorphic sites of the binding cleft formed by the HLA class II molecules are shown in figure 1.2.

Figure 1.2: Polymorphic sites in the peptide-binding groove of MHC class II molecules.



The ribbon diagram shows the α carbon backbone of the MHC class II molecule. The most polymorphic locations in the antigen-binding cleft are highlighted in red.

The association of HLA class II with disease

Susceptibility to almost all autoimmune diseases is strongly influenced by a multitude of genes (67;68) and studies have demonstrated that autoimmune disease is more prevalent in people with certain MHC types (69, 70). The binding grooves of the different allelic forms of a particular HLA molecule vary in their ability to bind a given peptide, depending on its net charge, size and hydrophilicity (71), and so determine an individuals' immune responsiveness to particular antigens, including autoantigens.

Thus, certain HLA genes may confer susceptibility to certain kinds of autoimmunity. And, more specifically, it seems determinants of specificity may be in the form of short stretches of amino acids in specific locations. Such limited sequences are referred to as shared epitopes, such as that recognised in RA (72;73). Further more, studies at the molecular level have identified single amino acid residues at specific locations of the binding groove as candidates associated with disease susceptibility.

For many autoimmune diseases HLA associations have been characterised (74). The contribution of MHC genes predisposing individuals to insulin dependent diabetes mellitus (IDDM) is undisputed (75). The disease appears in individuals, usually before the age of 20 and is the result of the selective destruction of the insulin-producing cells in the pancreas (74). HLA alleles DQB1*0302 and DQB1*0201 are associated with a strong predisposition to the disease, while DQB1*0602 is regarded to protect or confer resistance. Analyses for polymorphic residues shared among alleles associated with the disease have identified a correlation of position 57 of the DQ beta chain with disease susceptibility or resistance. The alleles associated with the disease posses either alanine, valine or serine at position 57. In contrast, those alleles found with decreased frequency in IDDM have an aspartic acid residue at this locus (74). The frequency of alleles possessing aspartic acid varies considerably with ethnicity and studies have shown a relative difference in the occurrence of IDDM between ethnic groups (74).

Genetic studies have linked a predisposition of RA with the HLA-DRB1 locus. Genetic analysis has revealed that HLA-DRB1*0401 and 0404, and DRB1*0101 confer genetic predisposition to RA (76;77). These alleles share sequence homology, within the third hypervariable region of the DR beta chain, and have given rise to the 'shared epitope' hypothesis (72;73). Pemphigus Vulgaris is an autoimmune condition of the skin and is slightly more common in Jews, than other ethnic and racial groups. An increased frequency in HLA DR4 has been identified in Ashkenazi Jews with pemphigus vulgaris, compared with healthy controls (74). Studies also suggest that the HLA DQ locus is important in susceptibility to this condition where an association with HLA DQ1 has been identified (74).

HLA associations with myasthenia gravis have also been reported, however are not as well established as those associations already mentioned. An association with HLA

DR3 in young Caucasian myasthenia gravis patients has been identified (75). Studies in myasthenia gravis patients have also identified an association with HLA DQ8 and DQ6. Mouse models have been used to demonstrate the role of class II molecules in experimental autoimmune myasthenia gravis. An evaluation of the role of DQ genes in this disease used transgenic mice expressing HLA DQ8 and DQ6. It was demonstrated that the HLA DQ locus affected the incidence and severity of experimental autoimmune myasthenia gravis, which suggested a direct role of particular HLA class II molecules in the modulation of the disease (75).

HLA associations have also been identified with multiple sclerosis; a chronic immune mediated demyelinating disease of the central nervous system. Association with the HLA DR2/ DQ6 extended haplotype in Caucasian multiple sclerosis patients have been well established. Correlation with MHC class II molecules is ethnically variable and association of multiple sclerosis with HLA DR1, DR3 and DR4 has been identified in other ethnic populations (75). HLA associations with other autoimmune conditions include SLE with DR2 and DR3, Hashimotos thyroiditis with DR3 and DR5 and Graves' disease with DR3 (74;75).

Associations of HLA with SSc

In SSc a number of HLA class II alleles have been associated with increased frequency of SSc disease, including DR2, DR3 and above all DR5. Stronger associations have been identified when clinical and autoantibody subgroups have been considered, however there is still no absolute pattern of association. There is an apparent variability in associations reported from different centres. Possible reasons for discrepancies include ethnic variability, clinical heterogeneity, and environmental factor diversity in different regions or localities. A comparison of HLA/SSc association studies from ethnically different populations are reviewed by Reveille '95 and Briggs and Welsh '91 (26;78), respectively. Allele and amino acids at particular loci associated with the three main serological groups in SSc subsets are presented in table 1.3.

Table 1.3: Reported HLA associations with SSc and major SSc serological subgroups

SSc serological subgroup	Major HLA associations	Associated amino acid residues at specific loci
Overall SSc	DR5	-
ATA	DR5 (DRB1*1101-1104) DR2 DQB1*0302, 0601	DQB1: Y: 30, DQB1: Y: 26, DRB1: FLEDR: 74-248
ACA	DR1 DR4 (DRB1*0403, 0407) DR5 DQB1*0501, 0301	Lacking DQB1: leu: 26
ARA III	DQB1*0201 (Single report).	-

HLA class II alleles and amino acid loci most commonly reported in association with SSc and major SSc serological autoantibody groups. DQB1: Y: 30: a tyrosine residue at position 30 of the outer most domain; DQB1: Y: 26: tyrosine at position 26 of the second hypervariable region in the β1 domain of the DQB1 gene; DRB1: FLEDR: 67-71; amino acids at positions 67-71 in the β1 domain of the DRB gene; DQB1: leu: 26: Lacking the amino acid leucine at position 26 DQB1 β1 domain. (25;63;64;79;80;80-86).

Due to the strong linkage disequilibrium that exists between the HLA complex genes the precise contribution of individual loci to disease remains difficult to dissect. In SSc, associations have been found with HLA-DRB1 and DQB1 alleles implying that both HLA DR and DQ genes control the autoimmune response in SSc (85).

Distinct evolutionary patterns in HLA class II allele frequencies are evident in ethnically diverse samples (87). The different prevalence of autoantibodies observed in separate ethnic groups (23), may be explained by the differences in HLA repertoire. This would support the role of HLA in SSc autoimmunity. As genetic techniques grow more sensitive and sophisticated the number of polymorphisms at each locus is steadily increasing, giving rise to more potential associate alleles. HLA associations may grow stronger still, with division of ethnic and racial groups studied, and consideration of not only the major autoantibody groups, but the more intricate autoantibody combinations characteristic of SSc.

Hormonal influences

As discussed earlier the majority of SSc patients are female, and the male: female variation is greatest during the childbearing years. The difference in gender appears to be attributable to female hormonal milieu or pregnancy-related events (20;72). It is thought that the sex hormones may influence immunological mechanisms, for example, it has been suggested that high concentrations of estrogens, as found during pregnancy, may inhibit T suppressor cells and increase B cell differentiation (88). They are also believed to affect cytokine production and vascular adhesion molecule expression (89). Masi *et al.* have reviewed hormonal contribution to the aetiology of SSc (88).

Microchimerism

It is now recognised that during the course of normal pregnancy, foetal cells routinely pass into the maternal circulation (90), where they may last for several decades (91). The presence of cell populations in the body, which are derived from a different individual, is termed chimerism. Microchimerism, which is described here, denotes low level of chimerism (90). Exposure to foetal cells represents an immunologic event because these cells express gene products that are inherited from the father, and are thus foreign to the pregnant women. Male DNA, identified by a Y-chromosome specific sequence, has been found in significantly higher concentrations in the blood of SSc patients who have had male offspring, compared to similar women who did not have SSc (92). Microchimerism has also been detected in the actual target organ in SSc. Artlett *et al.* found male DNA present in SSc skin biopsies of eleven SSc patients compared to none of the normal controls (93).

SSc has similarities with a known condition of human chimerism: chronic Graft Versus Host Disease (cGVHD), which occurs in some recipients of allogeneic marrow transplantation. SSc and cGVHD share clinical and serological similarities. Both conditions show progressive skin changes, the same internal organs are often targeted, particularly the gut and lungs, and often occur in overlap with Sjogren's syndrome and myositis (90). ATA and anti-Pm-Scl autoantibodies have also been described in some patients with cGVHD (94). However, striking differences between these two conditions are also noted; Raynaud's disease and renal involvement are rare manifestations in cGVHD (95). Chronic GVHD is well described in immunocompetent individuals who have received blood transfusions from family members who are HLA compatible due to

homozygosity (92). Moreover, it has been hypothesised that HLA compatibility of a child is associated with subsequent SSc in the mother (96). These observations support the hypothesis that microchimerism may be involved in the pathogenesis of SSc.

Animal Models

There are now several animal models resembling SSc that provide evidence of a genetic contribution to the disease. Such models have implied the MHC and other genes in its pathogenesis.

Tight skin mouse (TSK 1) model

The most extensively studied animal model of scleroderma is the tight-skin 1 (*TSK1*) mouse model. Mice heterozygous for the autosomal dominant mutation phenotypically and serologically express features of human SSc. *TSK1* have thickened tight skin, and show typical visceral changes mainly to the heart and lungs. ANA, including ATA have been reported in 50% of mutant animals (97). Genetic work on this model has linked the mutation to mouse chromosome 2, a region synonymous to human chromosome 15q (98). Work by Siracusa *et al.* refined the location of the mutation, mapping it to the fibrillin 1 gene, and demonstrated duplication within the gene in the *TSK1* model (99;100). The work implies that the ECM gene is a potential candidate in the pathogenesis of SSc.

Tight skin mouse (TSK 2) model.

The tight skin 2 (*TSK 2*) mutation represents an animal model of cutaneous fibrosis similar to *TSK 1* which, displays both fibrotic and inflammatory alterations similar to those seen in SSc (101). The mutation appears as a result of administration of the mutagenic agent, ethylnitrosourea, and, in contrast to the *TSK 1*, resides on chromosome 1. Using microsatellite markers and gene probes Christner *et al.* have localized the *TSK 2* mutation to a two cM region of mouse chromosome one, and suggest Col3α1 as a candidate gene (102).

The avian model

University of California, Davis, (UCD) line 200 white leghorn chickens spontaneously develop a syndrome which shows the entire spectrum of clinical, histopathological and serological features of SSc (52). Disease is evident as comb inflammations and lesions,

dermal fibrosis, and distal polyarthritis within six weeks of hatching (103). Birds surviving for longer than two months show progressive development of oesophageal fibrosis and mononuclear infiltration of the heart and kidney and some ANA (103). Mating experiments of UCD line 200 chickens with F1 cocks and backcrosses with other inbred strains have demonstrated varying penetrance of disease. These results indicate the presence of genes (possibly MHC) that modify the penetrance of presumed major genes (104).

Further genetic associations

Association of *null* alleles of the C4A and C4B loci with SSc, have also been reported. It is thought that a consequent deficiency in C4 particles may result in inadequate immune response, which may give rise to excessive inflammation and possibly lead to autoimmunity (105). The C4A and C4B genes are located between the regions of the MHC class I and II, and are shown in figure 1.1. Close proximity of the MHC and complement genes means that they are in linkage disequilibrium. It has thus been suggested that some apparent SSc-HLA associations may in fact be due to complement deficiency genes (106). In Caucasian SSc patients the haplotype carrying HLA DR3 has been associated with the C4 null allele (C4A*Q0) (106;107). In Japanese, associations with disease and autoantibody subgroup have been reported, where C4AQ0 was significantly increased in lcSSc and SSc patients without ATA (108).

Advances in genetic technology have given rise to an increasing number of studies employing the use of microsatellite markers to investigate further SSc candidate genes. The rational behind the use of microsatellite markers to investigate disease associated genetic polymorphism is centred on the principle of linkage disequilibrium (for review see Vyse and Todd '96) (109). Microsatellite markers consist of 2, 3 or 4 repeated nucleotides and are designed to be located amid, or within a 1cM distance, of the candidate gene of interest. If an association exists between a marker and SSc, because the marker has been selected specifically to be in linkage disequilibrium with the specific candidate gene, any association thus indicates that the gene is associated with SSc. This also provides evidence that a polymorphism within the chosen gene may be involved in SSc susceptibility.

Susol *et al.* used microsatellite markers to investigate SSc genes involved in fibrosis. Associations between SSc and microsatellite markers for transforming growth factor beta (TGFβ) 1, 2 and 3, B chain of platelet derived growth factor (PDGFB) and tissue inhibitor of metalloproteinase-1 (TIMP1) were identified, from which it was concluded that polymorphisms within these genes may exist that predispose individuals to SSc, and may even be useful in predetermining the extent and severity of fibrosis (110-112). However, associations of microsatellite markers near to the TGFβ1, latent TGFβ1 binding protein, and TGFβ receptors I and II genes were not supported in a study of native American SSc patients (113). Associations of TNFα and TNFβ microsatellite polymorphisms have also been reported, and TNFα13 has been proposed as a genetic marker for SSc patients with ATA (114).

Intragenic polymorphisms have also been investigated and associations have been reported. In a study of 230 SSc patients, Fanning *et al.* investigated polymorphisms of TNF-RII and, TNF/LTα genotype in 230 SSc patients. Clinical subgroups, lcSSc, dcSSc and pulmonary fibrosis, were considered, and associations with particular TNF and TNF-RII genotype combinations were identified (115). In Choctaw SSc patients a number of single base polymorphisms within the fibrillin-1 gene have been identified (116), and microsatellite markers near the fibrillin-1 gene have been found to be associated with SSc (117). More recently these researchers further investigated an Amerindian haplotype HLA DR2 (DRB1*1602) previously associated with SSc. Using polymorphic microsatellite markers, they identified an extended haplotype (HLA DR2 (DRB1*1602, DQA1*0501, DQB1*0301, DPB1*1301) that was significantly associated with SSc in the American Choctaw. When the regions immediately flanking the HLA region were investigated no other significant associations were found (118).

With advances in genome science there is fast mounting evidence of a genetic contribution to SSc susceptibility, which appears to extend beyond the HLA.

Family studies and exclusive populations

Over 99% of SSc patients report no first-degree relative who has been diagnosed with SSc and 98% report no other affected family member of any degree of blood relation (20). However, although such cases are rare, there are reports in the literature describing multi-case SSc families. Twin studies are of particular relevance to this issue and

provide an astute means of investigating the relative contribution of genetic and environmental factors to the aetiology of SSc.

Feghali *et al.* studied 34 twin pairs, one or both of which had systemic disease. The overall concordance rate for SSc in these twins was estimated to be 5.9%, with no significant difference in the rate between identical or fraternal twins. In contrast, concordance for the presence of ANA was 100% in identical and 63.6% in fraternal twins. In the same study, HLA typing confirmed previously reported autoantibody associations in the SSc patients. Of note though, none of the healthy twins displayed SSc-specific autoantibodies (119). In a study of three monozygotic twin pairs, who were discordant for disease, autoantibodies were identified in each affected twin. Genotyping revealed the expected HLA allele, for the relative autoantibodies identified (120). HLA association with autoantibody, in the diseased twin, supports the hypothesis of MHC genes contributing to disease susceptibility. However, discordance for disease between the twin pairs implies that additional factor(s), possibly environmental, are also important in SSc aetiology. Similar findings of ANA positivity have been reported in the spouses of scleroderma patients (121). A study of SSc patients, which identified eleven SSc patients with ACA, also identified ACA in two relatives of the probands. The two relatives, one identical twin sister and one sister, both had SSc (122). On consideration of the HLA background of these individuals, McHugh *et al.* concluded that the presence of at least one HLA DQB1 allele encoding for leucine of position-26 of the first domain appeared necessary, but not sufficient for the generation of ACA (122).

As discussed earlier, the Full-blooded Choctaw Native Americans, residing in South-Eastern Oklahoma are a population of individuals who demonstrate the highest incidence of SSc. Indeed as Harley and Neas said "Something about the environment of Oklahoma or the genetic composition of the Oklahoma Choctaw must be increasing the risk of SSc" (30). Thus, such exclusive populations provide a unique means of research into elucidating the aetiopathogenesis of SSc. The Native American Choctaw share a conserved gene pool and a genetic haplotype has been identified in the Oklahoma Choctaw as a significant risk factor for SSc (31;123). However, the frequency of this haplotype, observed in another group of Choctaws living in another state, was comparable. These results strongly suggest the role of the environment in the

development of SSc, however as yet a significant environmental risk factor has not been revealed (31;123).

Population, family and twin studies have shown that genetic factors exert a significant effect on predisposition to autoimmune disease (124), and provide evidence to support the concept that inherited genetic factors play a role in the development of SSc, and possibly the ability to produce certain autoantibodies. However they also suggest that a major factor in disease development involves an acquired environmental exposure, the nature of which remains indeterminate.

Cancer

There are reports to suggest that there is an increase in cancer among patients with SSc (125;126). Studies have indicated possible associations between SSc and a variety of malignancies, particularly those of the lung (125;126). Older age of diagnosis of SSc has been found as a significant risk factor for cancer (126), and associations have been made with SSc specific autoantibodies. ACA, independent of titre, has been identified as a significant risk factor for cancer in a Japanese SSc population. (127). ATA has also been associated with the development of cancer in SSc patients, and has not been associated with cancer in patients without scleroderma (128). In a report of two cases of SSc patients who developed adenocarcinoma of the lung, ATA titre levels were increased considerably at the time cancer was diagnosed, and were reactive with multiple epitopes of topo-I (129). Some topo-I fragment-specific reactivities detected after the diagnosis of cancer had not been present before. One explanation for this, as suggested by the group, could be representative of epitope spreading (129).

1.6.2: Environmental related factors

An increasing number of environmental factors have been proposed to contribute to the induction of autoimmune disease and need to be considered in the aetiology of SSc. Such factors include exposure to various chemical and microbial agents, and biochemical environments.

Exposure to environmental agents

Several environmental agents are now recognised as predisposing or eliciting factors in some patients with SSc. Exposure to some environmental agents is thought to cause tissue damage, which may result in the release of self antigens; making available cryptic self peptides. These agents mainly comprise silica dusts, organic solvents and some drugs, and also include those listed in table 1.4.

Table 1.4: Environmental agents considered as predisposing or eliciting factors in some patients with SSc

Environmental agent	*Details*
Chlorinated hydrocarbons	Trichloroethylene, perchloroethylene
Organic solvents:	
Hydrocarbons	Aromatic and aliphatic hydrocarbons
Chlorinated ethylenes	Vinyl chloride. Possibly exposure to unreacted vinyl chloride monomer
Polymerisation of epoxy resins	Possible causative agent; Bis(4-amino-3-methylcyclohexyl)methane
Drugs	Some appetite suppressants Pentazocine, cocaine, bleomycin
Silica dusts	Exposure such as inhalation from mining
Silicone	Cosmetic breast surgery
Quartz/metal dust	Uranium mining

Haustein & Ziegler ('85) (130), Silman & Hochberg ('95) (131)

The majority of these substances are reported to induce a scleroderma-like disease (SD), which can be distinguished from idiopathic SSc in a number of ways. One distinct difference between idiopathic and environmental SSc resides in the sex ratio, which is 1:1 in the former (132). The extent and type of skin involvement in SD is more generalised, often with joint contractures and fibrotic nodules (133). Manifestations often involve the liver, kidney, nervous system and muscles as a consequence of toxic damage by the environmental agent (133). There is usually cessation or reversibility of the disease after early discontinuation of the exposure. Serologically there is an absence of autoantibodies in SD patients, with the exception of SD induced by silica or silicon.

Silica and silicone

Crystalline silica (or quartz) is an abundant mineral found in sand, rock and soil. Reports of SSc associated with silica dust mainly detail miners, quarrymen, sandblasters and sandstone sculptors and grinders (131). Individuals in these occupations have a higher risk of developing an SSc disease, clinically indistinguishable from idiopathic SSc, which is thought to occur as a result of inhalation of silica particles of respirable size (<10uM) (134). The relative risk of developing SSc for silica dust-exposed individuals has been estimated at 74-fold compared to the normal population over 40 years of age (133). The precise mechanism by which silica causes SSc is unknown, but it is thought that phagocytosis of silica crystals by pulmonary alveolar macrophages, and the subsequent spread with phagocytosis by endothelial cells, results in a cycle of endothelial and macrophage activation; thus stimulating cytokine release, leading to the vascular, immune and fibrotic features characteristic of SSc (discussed earlier) (132;134). Whilst ATA are the predominant autoantibodies present in silica-associated SSc (135), ATA and ACA have been identified in silica-exposed patients and are associated with the expected clinical subtype of disease (133;136).

Silicone breast implants have also been implicated as a causal agent of SSc. Although this remains a debatable topic, there is literature to support such an association. Two case reports describe the onset of SSc following silicone gel breast implantation (137;138). Both reports describe disease regression in these patients after elective removal of the implants. Additionally, in the later study, the remaining three women in the study who did not have their implants removed, died secondary to progressive visceral involvement (138). Maclaughlin *et al.* reported a significantly increased incidence of SSc in Denmark in women after breast implant surgery (139), however such associations are not always supported (140).

In trying to explain why some individuals exposed to silica develop disease and others do not one may again begin to consider immunogenetic background. HLA DRB1*0301 has been identified as a susceptibility component in ATA positive, silica exposed individuals (141). Also, in post implant SSc patients, a significantly low frequency of the amino acid residue leucine at position 26 of the first domain of the HLA-DQB1 allele was observed. These results were consistent with those reported for Caucasian women with idiopathic SSc and ACA autoantibodies (142). Both studies conclude that

whilst the initiating event for the development of silica associated SSc is distinct from that of idiopathic SSc, it is not clear whether the ensuing diseases are distinguishable (141;142).

Exposure to solvents and related compounds

Exposure to solvents has been implicated as a causal factor in the development of SSc since the first description of such a case by Rein in 1957 (143). Since then, many case reports have been published associating the exposure to organic compounds, particularly organic solvents with the development of SSc and are reviewed by Silman and Hochberg ('96) and D'Cruz (2000), (131;132), respectively.

A scleroderma-like disease has been well described in workers exposed to un-reacted monomer of the chlorinated ethylene, vinyl chloride (VC), whilst cleaning VC polymerisation reactors (130). It has been hypothesised that exposure may lead to the formation of epoxy intermediate products (epoxides/peroxides) with the liberation of free radicals. These products are detoxified, *in vivo*, by molecular derangement or, by binding to proteins or nucleic acids, giving rise to structurally altered proteins, which, it is thought may induce a pathologic (auto)immune response in genetically susceptible individuals (130;133), as discussed earlier. An increased prevalence of HLA DR3 and DR3/B8 haplotype has been reported in patients with VC disease (144).

Other case reports have implicated solvents with a similar chemical structure to VC, such as perchlorethylene and trichloroethylene (145). Trichlorethylene penetrates the skin and the metabolites are neuro- and hepatotoxic, resulting in a variety of symptoms including scleroderma-like lesions of the skin (130). Contact with aromatic and aliphatic hydrocarbons, principally toluene, benzene, xylene and white spirits, and also the vapour of epoxyresins, have also been associated with scleroderma (130-132).

Drug induced scleroderma

The development of scleroderma has also been reported in some patients following the use of specific drugs. These include the long-term use of appetite suppression drugs and the antitumour agent, bleomycin. Again, the mechanism by which disease occurs is not clear, however, bleomycin is known to stimulate collagen production by normal skin fibroblasts (146). The development of scleroderma, in young men, following the use of

cocaine has also been reported (147). Cocaine is known to cause vasoconstriction and it is hypothesised that this may act as the first step in the disease pathogenesis in these patients (131).

A genetic determinant has not been associated with scleroderma development following drug use *per se* (148), however genetic associations have been reported in other autoimmune conditions. Work at our institution identified HLA DR4, DR2 and the presence of at least one HLA DQB1: Y: 30 allele as a susceptibility factor for developing a lupus-like syndrome following long-term use (>6 months) of minocycline (149).

Microbial agents and molecular mimicry

One hypothesis of autoimmunity proposes that it develops as a result of stimulation by a microbial product (150). This concept suggests that a microbial peptide may share structural and sequence homology with a self-peptide and can stimulate pathogenic self-reactive specific T-cells to cause autoimmunity. This phenomenon is referred to as 'molecular mimicry'. There is evidence to suggest that molecular mimicry may play a role in the development of the autoimmune response in some SSc patients. The B cell epitope comprising amino acids 121-126 was reported to have homology to the UL70 protein of human cytomegalovirus by Muryoi *et al.* ('92). This lent further support to the observation by the earlier report by *Maul et al.* ('89), who identified homology of amino acids 741-746 of the same protein with the $p30^{gag}$ retroviral protein (151). Additional regions of homology between some SSc autoantibody targets and viruses have been reported and are reviewed by White '96 (37).

Free radicals, cryptic peptides and apoptosis

Endothelial damage, caused by ischaemic reperfusion, has largely been attributed to free radicals. Ischaemia, experienced during episodes of Raynaud's, or by exposure to the environmental agents implicated in the pathogenesis of SSc discussed earlier, are thought to give rise to free radicals. During ischaemia, xanthine dehydrogenase may convert to xanthine oxidase, which gives rise to the release of free radicals (O_2-and H_2O_2) (152). Endothelial cells are thought to be susceptible to superoxide injury during reperfusion. Free radicals are believed to damage capillary walls by peroxidation of lipid components of the endothelial cell membrane and/or degradation of collagen and

hyaluronic acid, components of the basement membrane (152). Endothelial damage is hypothesised to lead to a chronic (auto)immune response in susceptible individuals culminating in SSc (152) as discussed earlier. Optimum treatments for Raynaud's is an active area of investigation (153), and therapeutic intervention with free radical scavengers, such as allopurinol, has been suggested to help slow the progression of SSc, if such susceptible individuals could be identified.

The generation of cryptic epitopes forms the basis of one explanation for the break in tolerance and consequent autoimmunity culminating SSc. As mentioned earlier, Casciola-Rosen *et al.* hypothesised that reactive oxygen species arising from ischaemic reperfusion may, in the presence of appropriate metals, induce novel fragmentation of autoantigens, (specifically Topo I and RNA polymerase II), resulting in immunocryptic epitopes with the potential of initiating autoimmunity (43). During apoptosis, many of the autoantigens targeted in the SSc autoimmune response become concentrated in surface blebs and are cleaved, by granzyme B *in vitro*, and, during cytotoxic lymphocyte granule-induced death, generating unique peptide fragments (154). It is hypothesised that the unique peptides may be internalised by APCs and presented, causing the activation of autoreactive T-cells and autoimmunity (155).

1.7: The present study

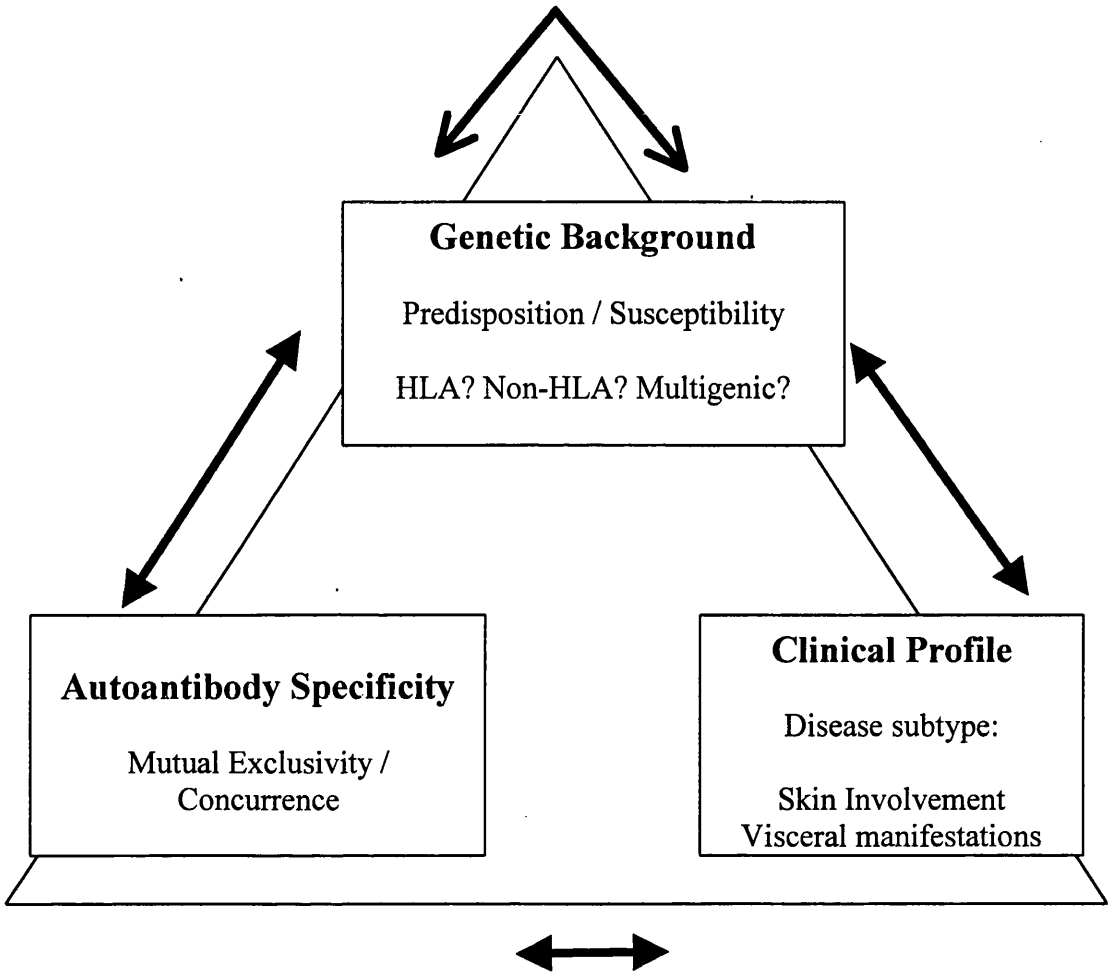
Whether in fact autoantibodies are agents of pathology or not, remains a debatable topic. However, autoantibodies are certainly markers of disease in SSc, and are thus a valuable source of information with regard to the pathogenic mechanism culminating SSc and autoimmune disease. Currently, serological autoantibody identification of SSc patients is one of the most useful diagnostic and prognostic tools available. As discussed, a number of ANA, are specific for SSc and are indicative of disease subtype and manifestation. A degree of autoantibody mutual exclusivity is also recognised: giving rise to the three, so called, major serological groups. Further autoantibody specificities are characteristic of SSc and although not considered disease specific, they have been associated with patterns of disease development and may indicate potential cases of disease overlap.

Genetic associations are also recognised in SSc and as discussed, HLA allele and amino acid loci associations with autoantibody specificity have been reported. However, although some broad correlations have been established, this area tends to fall subject to a degree of ambiguity. Racial and ethnic variations are now appreciated to be a contributory factor to this, and need to be considered, particularly when population comparisons are to be made.

1.8: Study Aim

In the present study, an investigation into the autoantibody, clinical and genetic status of SSc patients was undertaken, with an aim to further identify the associations of each of these aspects, with SSc, and to investigate a potential relationship *between* these three factors. This idea is represented in figure 1.3:

Fig 1.3: Genetic-Autoantibody-Clinical Profile Triad



A representation of the proposed *'Genetic-Autoantibody-Clinical profile'* triad-relationship, hypothesised to underlie the pathogenesis of SSc and investigated in this thesis.

CHAPTER 2

MATERIALS AND METHODS

2.1: PATIENTS AND CONTROLS

For this study four main groups of individuals were included:

1. Two hundred and twenty nine British Caucasian SSc patients attending; the RNHRD CTD clinic, Bath (n=100) or the Hope hospital, Salford (n=129)
2. Forty five South African Black SSc patients attending a tertiary hospital on the Witwatersrand, South Africa
3. Control cohort one: 250 normal, healthy, 'control' individuals attending the Southwest region blood transfusion centre.
4. Control cohort two: 196 normal healthy 'controls' from the Norfolk family Health Service Authority Register

2.1.1: Clinical Diagnosis

A clinical diagnosis of SSc was assigned when the minimum requirements, defined by the ARA preliminary criteria for the diagnosis of SSc, were satisfied. The criterion, published by the ARA, provides a standard for definite or certain disease in order to permit comparison of groups of patients from different centres. The published criteria are accepted as the worldwide standard for research purposes (156).

ARA preliminary criteria for the diagnosis of SSc

Either the major criterion or at least 2 of the minor criteria must be met for a diagnosis of SSc (97% sensitivity and 98% specificity).

Major criterion; scleroderma proximal to the metacarpophalangeal joints

Minor criteria; i) sclerodactyly

ii) Digital pitting scars of the fingertips, or loss of substance of the distal finger pad

iii) Bibasilar pulmonary interstitial fibrosis

2.1.2: Disease classification and scoring

Disease 'subtype' classification

Throughout this study disease has been classified as a subtype, either limited cutaneous SSc (lcSSc), or, diffuse cutaneous SSc (dcSSc) (2). LcSSc was defined as cutaneous sclerosis not extending proximal to the elbows or knees, with or without facial involvement, while dcSSc was defined as cutaneous sclerosis extending proximal to the elbows or knees.

Scoring system for skin involvement

The disease subdivision described above was further classified, based on the extent of cutaneous involvement, according to a modified version of Barnett's classification system (16). LcSSc patients were assigned as either; type 1) Sclerodactyly only or, type 2) Skin involvement limited to the hands, forearms, face and neck, and dc-SSc patients were classed as type 3) Diffuse skin involvement

Diagnosis and scoring of pulmonary fibrosis

Pulmonary fibrosis was assessed by; measuring forced vital capacity (FVC), diffusing capacity (DLCO), and/or scarring on chest X-ray. FVC is obtained from maximal forced expirations into a recording spirometer (and compared to predicted values according to age, sex, race and height). Any significant shadowing of the lungs characteristic of bibasilar pulmonary fibrosis, apparent by X-ray examination, was also recorded, according to the severity scoring system as follows:

Score	*Test Result*
0	Normal diffusion capacity
1	DLCO 70-80% predicted, FVC 70-80% predicted, bibasilar dry crepitations (discontinuous dry crackles, usually bilateral, almost exclusively on inspiration, which indicates interstitial lung disease)
2	DLCO 50-69% predicted, FVC 50-69% predicted. Pulmonary hypertension (peak systolic pressure 30-59mmHg)
3	DLCO <50% predicted, FVC<50% predicted. Pulmonary hypertension (peak systolic pressure 60+ mmHg)
4	Oxygen required

2.2: DNA BASED TECHNIQUES

2.2.1: Isolation and quantification of DNA from whole blood

Isolation of DNA from whole blood

DNA was prepared from whole blood, which had been collected into tubes containing EDTA. After centrifugation at 1200r.p.m (Beckman GPR centrifuge) for 10min, the 'buffy' layer (containing the white cells) was carefully pipetted into a clean tube. Eight ml of red cell lysis buffer (0.144M NH_4Cl, 10mM $NaHCO_3$) were added and after 20min the tube was centrifuged as before. The red cell lysate was removed and the pellet resuspended in 3ml nuclei lysis buffer (10mM Tris HCl pH8.2, 0.4M NaCl, 2mM Na_2EDTA pH8.0), 100µl proteinase K (10mg/ml in buffer; 2mM Na2EDTA, 1% w/v SDS), and 100µl 20% SDS. After the samples had been incubated in a water bath, for 3 hours at 55^{O}C, 1ml of 5M NaCl was added and the tube shaken vigorously for 20sec. The tube was centrifuged as before and the supernatant collected into a universal vial, (care was taken to leave the pellet undisturbed). To the supernatant 8ml of absolute alcohol were added and the contents mixed by repeated gentle inversion of the tube. The precipitated DNA was removed by winding onto the end of a sealed Pasteur pipette and excess alcohol squeezed out on the side of the tube. The DNA was transferred to an Eppendorf tube and dissolved in 100µl of double distilled filter purified water (ddH_2O) and stored at –25^{O}C. DNA was quantified by spectrophotometry, as detailed below.

Quantification of DNA by Spectrophotometry

Nucleotides in solution absorb light in the ultraviolet (UV) region of the spectrum, with a maximum absorption around a wavelength of 260nm. Proteins also absorb UV light but at the 280nm wavelength. Therefore, nucleic acids can be quantified, and the purity of a preparation estimated by measuring the absorption at both 260 and 280nm (spectrophotometry) and comparing the results to those obtained from pure solutions. The term optical density (OD) is commonly used instead of absorbance.

Preparation of samples for spectrophotometry

DNA samples to be quantified and assessed for purity, (typically 15 at a time), were removed from the freezer and allowed to thaw at room temperature. Eppendorf tubes were labelled appropriately and 250µl of ddH_2O were placed into each tube. Once thawed, the DNA samples were mixed thoroughly by vortexing for 20sec. A 2.5µl

aliquot of DNA sample was pipetted into each of the allocated Eppendorf tubes. Care was taken to dispense the entire sample from the tip by sucking the solution up and down a few times. (If the DNA sample was very viscous then 100-200µl of ddH2O were added to the sample, which was thoroughly mixed by vortexing and then placed in the fridge for a couple of hours. This procedure was repeated until the sample reached a 'workable' viscosity). Tubes were vortexed for 10sec and placed in the fridge for 30min.

Spectrophotometry of samples

The spectrophotometer (LKB, Blothrom, Ultraspec II) was calibrated for reading wavelengths of 260 and 280nm, using ddH_2O as the 'blank', according to the manufacturer instructions.

The first DNA sample was pipetted into a clean cuvette (Helm, model 105.201-QS) and placed into the spectrophotometer. The OD 260nm, (measure of nucleic acid content), was recorded first, followed by the OD 280nm (measure of protein contaminants). A third reading, the ratio OD of 260/ 280 indicated the purity of the sample. A reading of 1.8 identified pure DNA samples. Samples with a ratio of 1.8 (+/-0.1) were of sufficient purity for the work intended. Ratios of samples not falling within this range were purified, according to the procedure detailed below.

Calculation to determine the concentration and quantity of DNA

DNA concentration was calculated as follows:

$$\frac{\text{OD 260nm X 50 X dilution factor}}{1000} = X\mu g/\mu l$$

The quantity of DNA in a sample was calculated by multiplying 'X' from the above calculation by the total sample volume (Yµls).

Purification of DNA samples

A low OD ratio indicates protein contamination of nucleic acids. Any such contaminations were eliminated by treatment with phenol and chloroform as follows: To the DNA suspension an equal volume of phenol (Appligene, Oncor, France) was

added. The mixture was shaken in a closed tube until an emulsion had formed (this served to denature any protein). The tube was centrifuged for 15sec at 13000rpm (MSE microfuge), and the upper phase was transferred to a fresh Eppendorf tube, (the lower organic phase and interface were discarded appropriately). An equal volume of 1:1 mixture of phenol and chloroform (Appligene, Oncor, France) was added to the retained layer, which was then mixed, spun, and separated as before. To the subsequent retained 'upper' layer a 1/10 volume of three molar sodium acetate was added followed by 2 volumes of absolute alcohol (Sigma Chemical Co.). The tube was agitated and spun for a third time, after which as much fluid as possible was taken off with care taken not to disturb the DNA pellet. The pellet was washed with alcohol twice more and left to air dry. Once dry the DNA was dissolved in 100µl of sterile ddH_2O.

2.2.2: MHC class II HLA DRB1 and DQB1 typing by PCR-SSP

The polymerase chain reaction (PCR) is a technique for the *in vitro* amplification of specific DNA sequences by the simultaneous primer extension of complementary strands of DNA. Two primers, each complementary to opposite strands of the region of DNA, which have been denatured by heating, are employed. The primers are designed such that each primer extension (synthesis of a complementary strand in the 5' to 3' direction) reaction directs the synthesis of DNA towards the other. Reaction cycles result in the amplification of the region of DNA of interest (i.e. between the primers).

HLA-DRB1 and HLA-DQB1 alleles have been identified using a PCR based method with sequence specific primers (SSP).

Primers for the amplification of HLA DRB1 alleles

Ten 5'-primers and 16 3'-primers, combined in 20 primer mixes, were used for identifying HLA DRB1 alleles. Also in each PCR reaction a pair of primers specific for non-allelic sequences was included. The latter primers amplified the third intron of DRB1 genes and functioned as an internal amplification control (appendix II) (157;157;158;158).

Allele specific amplification of DRB1*04 alleles was carried out using one 5' primer and eight 3' primers combined in 8 (5'+3') primer mixes with control primers in each

reaction (113). Allele specific amplification of DRB1*01 alleles was established using four 3' primers and one 5' primer combined in 4 reactions (159).

Primers for the amplification of DQB1 alleles

Eleven 5' primers and eleven 3' primers combined in 14 primer mixes were used for the identification of DQB1 alleles. Two 5' and two 3' primers were combined to further distinguish between alleles HLA DQB1*0301 and 0304 (appendix II) (160).

Reaction Mixes

The PCR reaction mixtures (10µl) consisted of 300ng genomic DNA in 1µl reaction buffer (200mM $(NH_4)2SO_4$, 750mM Tris-HCl, pH9.0 at 25°C), $MgCl_2$ 1.5mM, 125µM of each dATP, dGTP, dCTP and dTTP, 0.25µM sequence specific primers, 0.15µM control primers (C3 and C5), 1.9µl sucrose cresol red (60% sucrose, 1mM cresol red), and 0.25 units Taq (Thermoprime +, Advanced Biotechnologies).

Cycling Parameters

PCR amplifications were carried out in a PTC-200 DNA Engine (MJ Research). DNA was amplified by 30 cycles at three temperatures, with an additional denaturing step and extension step. The process was started with a 2min denaturation at 96°C, followed by 10 cycles consisting of denaturation at 96°C for 25 sec, annealing at 65°C for 50 sec and extension at 72°C for 30 sec. The process was then 'stepped down' to denaturation at 96°C for 10 sec, annealing at 61°C for 50 sec and extension at 72°C for 30 sec for 20 cycles. The process was completed by a 10min incubation at 72°C followed by chilling at 4°C.

Agarose gel electrophoresis for visualisation of amplification

PCR products were visualised by horizontal electrophoresis in 2% ethidium bromide agarose gels. The gels were made by adding 3g agarose (Flowgen agarose) to 150ml 0.5 X Tris Borate EDTA (TBE) (45mM Tris Base, 45mM boric acid, 1mM EDTA pH8.0) and boiling the mixture in a Pyrex flask placed in a microwave oven, with occasional stirring, until the agarose had dissolved. After being left to cool to approximately 50°C ethidium bromide was added to a final concentration of 0.5µg/ml. The molten gel was

poured immediately into a gel tray and 15 or 20 well combs slotted into place. The gel was left to set.

The PCR reaction mixtures were loaded into the 4 X 1.5mm wells. The sucrose cresol red acted as loading solution. Gels (25x13 cm, 4x20 wells) were run at 150V in 0.5 X TBE buffer for approximately 45min. Gels were examined under UV illumination (UV transilluminator-UVP), recorded and documented using the computer photographic package 'Imigdoc'.

2.2.3: SSc candidate gene analyses using microsatellite markers

Research into the investigation of SSc candidate genes using microsatellite markers was carried out at the ARC epidemiolgy unit at the University of Manchester. The experimental procedures used for this work and described below, had previously been optimised by Elene Susol and colleagues.

Collection of DNA samples

DNA samples were collected from patients as described in method 2.2.1.

DNA Quantification

Semi-automated techniques for genotyping microsatellite markers require DNA of a known concentration, thus all DNA samples to be typed were firstly quantified by fluorimetry. This was done using the Hoefer Mini Fluorimeter (TKO 100), which allows the quantification of dsDNA by measuring excitation and emission spectra of a fluorescent dye (Hoechst 33258) in the presence and absence of dsDNA. Thus DNA concentration was calculated according to details set out in the manufacturer instructions.

Before each session it was necessary to calibrate the fluorimeter. Firstly the fluorimeter was zeroed using 2ml working dye solution (50ul H33258 stock solution in 5ml filtered 10 X TNE (100mM Tris, 10M EDTA. 1M NaCl pH7.4), 45ml filtered distilled water (0.45μm pore filter, Acrodisc) pipetted into a clean cuvette. Standard DNA (2μl of 1mg/ml calf thymus DNA; Clonetech) was then added to the cuvette, which, was

returned to the fluorimeter and the reading adjusted to 1000 (equivalent to a DNA concentration of 1μg/ml). This procedure was repeated 3-4 times until the DNA standard reproducibly read 1000.

PCR using microsatellite markers

Proteins involved in fibrosis that are thought play a role in the pathogenesis of SSc were investigated. Microsatellite markers mapping within or close to the corresponding candidate genes of interest had carefully been selected by colleagues in Manchester and are detailed in table 2.1.

Table 2.1: Microsatellite markers used in the investigation of SSc candidate gene polymorphism

Candidate gene	Micro-satellite Marker	Primer sequence	PCR product size (bp)	Fluorescent Dye
TGFβ3	D14S277	FP: TTGCTTTCACTCCCCATT RP: TGCACTTGAAGATTCAGATAAG	150-170	FAM
TGFβ1	D19S400	FP: ATGACAGCTCTAGGAAG RP: CGGTATGTCTTTATCAGCAG	195-230	FAM
PDGFB	D22S284	FP: GCTCTCTTGAGGTCGTTACA RP: ATGGGTATTTAACTTCTCTACA	76-112	TET
Col5A2	D2S389	FP: TAAAGCCTAGTGGAAGA RP:GCTGAGTTAACAGTTATCAACA	179-229	TET
PDGFB	*PDGFB	FP: AGAGGTGAATTTGCAAGTGA RP: AHGTGATGGTTATTACTGCAG	75-108	HEX
TGFβ2	D1S419	FP: CGCATGTAAGTTGGGG RP: AGCTGGTCCTGTCTGGAT	152-204	HEX
TIMP1	DXS426	FP: CCTTCATCTACCAAGATA RP:CTGCACTCCAGCCTGAATAA	271-317	HEX

Microsatellite markers used for the investigation of candidate 'fibrosis' genes. All markers were located within 1cM of the relative candidate genes with the exception of *PDGFB which was located within the gene. Forward primers were labelled with fluorescent dye (FAM; blue, TET; green, HEX; yellow, TAMRA; red). fp; forward primer, rp; reverse primer.

Reaction mix for PCR using microsatellite markers

PCR was carried out in a Hybaid Omnigene thermocycler using 96 well plates. The total volume of each PCR reaction was 10µl and the precise composition of each mix is detailed in table 2.2:

Table 2.2: Reaction mixes for PCR using microsatellite markers

Component	Stock concentration	Amount added to PCR	Final concentration/ amount in reaction mix
DNA	10ng/µl	5µl	50ng
Bioline NH_4 reaction Buffer	10x	1µl	1X
Bioline $MgCl_2$	50mM	0.3µl (0.2µl, D19S400 mix only)	1.5mM (1.0Mm, D19S400 mix only)
Bioline d'NTPs	50mM	0.5µl	0.1mM
Bioline Taq DNA polymerase	5 units/µl	0.04µl	0.2 units
Forward primer	50pol/µl	0.2µl	10pmol
Reverse primer	50pmol/µl	0.2µl	10pmol
Sterile betaine	4M	*2.5µl	1M
Sterile autoclaved distilled water		Make each reaction to 10µl, total volume	

Details of the composition of each single reaction mix for PCR using microsatellite markers. Sterile betaine was only added to reactions containing microsatellite marker D14S277 or D19S400.

Cycling parameters for PCR using microsatellite markers

Each reaction mix was covered with a drop of paraffin and plates were located into the PCR machine. PCR was carried out as follows:

1 cycle	45 seconds at 95°C	
35 cycles	45 seconds at 95°C	
	1 minute at 55°C *or*	56°C for D14S277 and D19S400
	45 seconds at 72°C	
1 cycle	5minutes at 72°C	

After the PCR procedure was complete, products of each microsatellite marker set for each individual patient were pooled together and analysed using ABI semi-automated gene scanner technology (detailed below). However, to determine the appropriate volume of each PCR product to be pooled, firstly, it was necessary to measure the PCR products' fluorescence intensity. This was done by selecting three random samples from each PCR and analysing them by running a 'test gel', using the ABI semi-automated gene scanner technology, as described below.

Genotyping using the ABI-Semi-automated gene scanner

Genotyping was carried out using an ABI Prism 377 DNA sequencer. Briefly, this procedure involves vertical polyacrylamide gel electrophoresis of fluorescently labelled PCR products. The products are detected by an argon ion laser beam and information is downloaded to a computer equipped with software for characterisation of PCR product size and allele identification.

Vertical polyacrylamide gel electrophoresis for ABI 377 DNA sequencer

Two glass plates were thoroughly cleaned with distilled water and 70% isopropanol using white tissue paper. 0.2mm spacers were placed between the plates, which, were then orientated correctly and assembled into the gel cassette. Care was taken to ensure that the bottom edges of the plates were even. A four percent acrylamide gel was made (30ml 4.0% acrylamide (Severn Biotech Ltd.), 240µl, 10% ammonium persulphate and

12µl of TEMED (Amresco)), and poured between the plates using a syringe. The flat edge of a 36-well comb was immediately positioned between the top of the plates and left for two hours to allow the gel to polymerise. Once the gel had set, the comb was removed, rinsed and the toothed edge was reinserted between the plates, down towards the top of the gel, thus creating wells into which the samples were to be loaded.

From this stage the operating instructions for the ABI prism 377 DNA sequencer were carefully followed, and the procedure is described below.

The gel cassette (containing the gel) was located in the ABI prism 377 DNA sequencer and secured. To ensure that the scanning region was clean a plate check was carried out. The buffer tanks were filled with 1xTBE (45mM Tris Base, 45mM boric acid, 1mM EDTA pH8) and the gel set to run until it reached 51°C.

Preparation of samples for loading into the gel

One µl of PCR product was added to 2.5µl of prepared loading solution (20µl of fragment size standard solution; Prism Genescan 350 TAMRA, N,N,N',N'-Tetramethyl-6-carboxyrhodamine, Applied Biosystems, with 80µl of loading dye; few granules of Blue Dextran 2000 in 80µl Ultra Pure Grade Formamide, Amresco). DNA was denatured by heating for 2min at 95°C and then samples were immediately placed onto ice.

Running the ABI Prism 377 DNA Sequencer Genescanner gel

Each sample was loaded into the wells and the gel set to run at 51°C for two hours, (3000V, 60mA, 200W), to allow the DNA fragments to separate according to size. The ABI gene scanner laser continuously scanned the lower portion of the gel. As each DNA fragment was scanned, the tagged fluorescent dye was excited and the light emission collected and separated according to wavelength onto a camera. Each fluorescent dye (TAMRA, TET, HEX and FAM) emits light at different wavelengths; so all four emissions could be recorded and deciphered simultaneously, with one pass of the laser. Information was processed electronically by the 'data collection programme' and downloaded into files for GeneScan analysis.

Genotyping of alleles

Firstly, it was necessary to size the DNA fragments. To do this, each lane of the gel was tracked on an image of the gel, generated by the GeneScan Analysis software. Subsequently, sample files and electropherogram plots for the DNA fragments in each lane of the gel were generated, and the TAMRA standard fragments were checked. This data was then used to size the microsatellite marker PCR products.

The software package, Genotyper was used to assign alleles, using the data, which had been generated by the GeneScan Analysis (previously). Microsatellite alleles, represented as peaks of fluorescence on the electropherogram, were sized (base pairs). Upper and lower size limits of each allele were defined and an allele identified according to a three-digit code (101, 102 etc.). A macro, which had been designed previously, was used, which automatically analysed all samples from gel runs. Two control samples were included on all gels to ensure consistency in genotyping by this method. However, all genotyping was checked manually to ensure that alleles had been correctly identified by the software (hence the term 'semi-automated genotyping'). Genotyping results were copied to Microsoft Excel spreadsheets for analysis.

2.3: TISSUE CULTURE TECHNIQUES

Basic Tissue culture

Standard aseptic practice was employed whilst working in the tissue culture laboratory and precautions appropriate for the materials being used were taken. Procedures were carried out in class II tissue culture cabinets with lamina flow of filtered air. Any non-sterile materials and solutions were autoclaved prior to use.

2.3.1: Preparation of tissue-culture media

Rosewell Park Memorial Institute (RPMI) media

A fifty ml volume of a 10X concentrate of RPMI media, without sodium bicarbonate or glutamine, was diluted in 375ml ddH_2O and supplemented with 15ml sodium bicarbonate solution (7.5% w/v), 5ml penicillin/streptomycin solution (10000 I.U./ml and 10000μg/ml respectively), and 5ml glutamine solution (200mM), (all Life

technologies Ltd, Paisley, U.K.). Double distilled H_2O was added to a final volume of 500ml for serum-free media. Alternatively 50ml of heat-inactivated foetal calf serum (FCS) were added for 'RPMI+10% FCS' media.

Methionine-deficient media

One pot of non-sterile powdered RPMI (Sigma Chemical Co. Ltd.); deficient in sodium bicarbonate, glutamine, methionine, leucine and lysine was dissolved in 775ml of filter-purified water, according to the manufacturers instructions. The solution was sterilised by passing through a bottle top filter (cellulose acetate membrane, 0.22μm pore size; Becton Dickinson Labware), attached to a vacuum pump. The medium was then supplemented with 30ml sodium bicarbonate solution, (7.5% w/v), 10ml penicillin/streptomycin solution (10000 I.U/ml and 10000μg/ml respectively), 10ml L-glutamine solution (200mM) (All; Life Technologies Ltd, Paisley, U.K.), and 5ml l-Leucine (200x solution for tissue culture; Sigma Chemical Co. Ltd.). The volume was made up to 1 litre with ddH_2O for 'serum-free methionine-deficient RPMI', or with 50ml dialysed FCS (d-FCS; prepared according to the method below), for 'Methionine-deficient RPMI + 5% d-FCS'.

Preparation of dialysed foetal calf serum

Preparation of dialysis tubing

A 900mm piece of dialysis tubing (Sigma Chemical Co. Ltd.) was washed under running tap water, for 3-4 hours to remove the glycerine coating. Sulphur compounds were then removed as follows; approximately 300ml of EDTA-bicarb, (200mM sodium bicarbonate, 5mM EDTA), was poured into a glass beaker and brought to boiling point on a hot plate. The tubing was plunged into the solution for 5min, and then rinsed with ddH_2O. The boiling and rinsing steps were repeated once more after which the tubing was used immediately.

Dialysis of FCS

Two knots were secured in one end of the tubing and FCS poured in from the open end until the tubing was approximately three quarters full. The open end was then similarly secured. After rinsing the tubing with phosphate buffered saline (PBS, prepared according to manufacturers instructions (Oxoid Unipath Ltd, Basingstoke, U.K.)) to

remove any spillages of FCS, the tubing 'sausage' was placed into a beaker containing 3l of chilled PBS. The beaker was placed on a magnetic stirrer at an ambient temperature of 4°C for 8 hours. Then PBS was exchanged for a fresh 3l of PBS, and the tubing left for a further 8-12 hours. Then the PBS was exchanged once more, as before, and left for another 8 hours. After a total of approximately 30 hours dialysis, the tubing was rinsed in PBS, cut open and the d-FCS tipped from the tubing into a clean 50ml polypropylene tube (Falcon brand). The d-FCS was immediately filter-sterilised by pushing steadily through a bottle top filter (pore-size 0.22μm: Millipore), into a sterile glass bottle, with a 10ml syringe.

2.3.2: Cell culture and maintenance

Initiation of cells

The non-adherent K562 cell line, derived from a human chronic myelogenous leukaemia, were recovered either from storage under liquid nitrogen in Bath or, if stores were depleted, from growing cultures supplied by ECCAC (No. 89121407).

Resuspension of frozen cells

Frozen vials of cells were rapidly defrosted in a water bath (37°C), and the cell suspension was then washed twice in warm RPMI + 10%FCS medium before culture.

Maintenance of cells

K562 cells were maintained in approximately 30ml RPMI + 10%FCS, in 75cm^2 tissue culture flasks (Falcon brand), between 8-30 million cells/flask, and incubated in a 5% carbon dioxide humidified incubator set at 37°C.

Freezing cells for storage

Approximately 100ml of confluent cells were needed for the preparation of five, 1ml aliquots of K562 cells for freezing. The appropriate volume of cells were pooled into 50ml polypropylene tubes and centrifuged at 1200r.p.m for 10min (Beckman/GPR). The supernatant was poured off and the cells resuspended in 0.5ml RPMI + 30% FCS and pooled into one tube. A small sample of the cells was put onto a haemocytometer and counted. After calculation the appropriate volume of RPMI + 30% FCS was added

to give a concentration of $10X10^6$cells/ml. An equal volume of 20% dimethylsulphoxide (DMSO) in RPMI + 30% FCS was then added to the suspension and mixed in (final concentration $5X10^6$cells/ml). One ml aliquots (approximately 5) were pipetted into cryovials, which were placed into a polystyrene box and frozen at -80°C for at least 10 hours prior to storage in liquid nitrogen.

2.4: SEROLOGICAL BASED TECHNIQUES

2.4.1: Preparation and storage of serum samples

Serum samples were obtained as follows: 7ml of blood was collected into a standard, glass blood sample tube. The sample was left to stand until the blood had clotted (approx. 30min), after which the tube was centrifuged at 3000r.p.m. for 10min (Hereaus Labofuge 6000 Centrifuge). The serum fraction of the sample was drawn off with a pipette and dispensed, in 1ml aliquots, into serum storage vials. Serum samples were stored at -20°C.

Detection of autoantibodies

The presence of antinuclear antibodies was studied by indirect immunofluorescence of Hep-2 cells (BioDiagnostics, Upton-upon-Severn, U.K.). The fine specificity of antinucleolar antibody was investigated by immunoprecipitation of ^{35}S-radiolabelled cell extract by SDS-PAGE. Ouchterlony double immunodiffusion was also used.

2.4.2: Indirect immunofluorescence

Serum was incubated against a fixed substrate to allow binding of autoantibodies to their respective antigen(s). A secondary antibody conjugated to fluorescein was then incubated against the substrate–antibody. Slides were mounted and studied under a fluorescence microscope. The presence of antinuclear antibodies were recognised by particular patterns of fluorescence.

Detection of antinucleolar and anticentromere autoantibodies

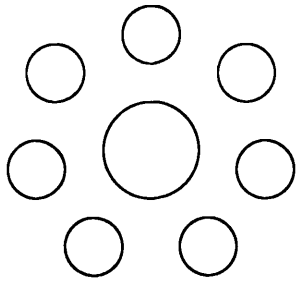
Sera were thawed at room temperature and diluted 1 in 40 with PBS (Oxoid Ltd., Hampshire, England). Refrigerated multiple well HEp-2 slides (Binding Site, Birmingham, U.K.) were placed in a humidity chamber and left to come to room temperature. Thirty µl of diluted serum were pipetted onto the wells and the slide returned to the humidity chamber and incubated for 30min. Slides were rinsed with PBS and then placed in a PBS bath for 5min. Slides were removed from the bath, carefully dried between the wells with a tissue and returned to the chamber. Twenty µl of anti-human polyvalent immunoglobulin fluorescein isothionate conjugate (FITC) (Sigma Chemical Co. Ltd., F6506) were diluted 1 in 150 with PBS and added to each of the wells. After 30min incubation in the humidity chamber, slides were washed as before and coverslips mounted. Slides were read under a fluorescence microscope to identify antinucleolar and anticentromere positive sera.

2.4.3: Ouchterlony double immunodiffusion

An antigen rich tissue extract and serum from a patient are allowed to diffuse towards each other in a horizontal agarose gel. When the patient serum contains autoantibodies, which recognise 'antigen' contained in the extract, then a line of precipitation is formed by the antigen/antibody complex (zone of equivalence).

An adaptation of the method described by Isenberg and Maddison ('87) was used (161). An $8cm^2$ glass plate was cleaned with ethanol and placed on a levelling table. Onto the plate 12ml of 1%, molten, agarose gel containing 5µg/ml penicillin/streptomycin solution (10 000 IU/ml and 10 000 µg/ml respectively) were poured. The gel was left at room temperature to set. Circular wells were punched, precisely, into the gel using cork borers, in the formation shown in figure 2.1:

Figure 2.1: Arrangement of wells punched into agarose gels

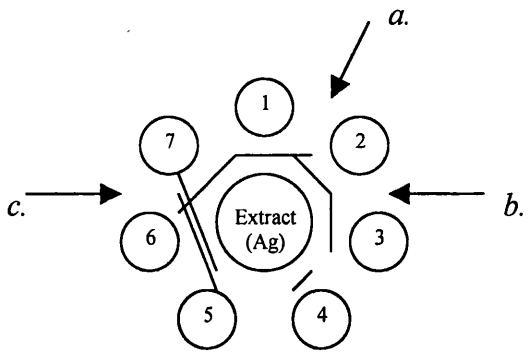


Seven wells of 4mm diameter evenly surrounded one central well of 7mm diameter. Each smaller well was equidistant from the other, and positioned 1.5mm away from the central well, to form 1 rosette. (When testing sera against topo-I extract (TIE) 6 smaller wells only were used).

Agarose plugs were removed by careful 'teasing out' using a capillary tube. Twenty µl of test or standard serum were pipetted into each of the small, outer wells, immediately followed by the addition of 80µl of tissue extract (20µl for TIE) to the larger central well. A petri dish lid was placed onto the levelling table, covering the plate and gel, and left for 12 hours.

The gel was viewed against a black background illuminated by a strong desk lamp. Gels were examined, after 12 and 14 hours incubation, and any precipitin lines on the gel were recorded and interpreted. Possible precipitin patterns and interpretations are shown in figure 2.2.

Figure 2.2: Interpretation of precipitin patterns



Possible precipitin patterns are represented as follows; *'a.'* represents a *line of partial identity*. This occurs when a particular antigen is recognised by Abs present in both sera, and a separate antigen is recognised by antibodies occurring in only 1 serum. *'b.'* shows *a line of identity*. This indicates that both sera contain Abs that recognise exactly the same antigenic particle. At *'c'* all lines cross, showing *lines of non-identity*, indicating that adjacent sera contain Abs of different specificities. The precipitin line beside well 4 represents a sera with a low titre of Abs. Repeating the experiment with more serum accommodated in a slightly larger well may allow for the identification of autoantibody specificity.

Each serum sample was tested against 3 tissue extracts, which contained a broad spectrum of antigens (rabbit thymus extract (RTE) (Pel Freez Ltd, Arkansas, U.S.A/ Bradshaw Biologicals, Market Harborough, U.K.); containing U1RNP, sm, La and Pm-Scl antigens; calf thymus extract (CTE), prepared in house by BIRD Diagnostics, (Bath, U.K.), which contained Jo-1, and an Scl-70 extract (TIE) (Biodiagnostics Ltd), which contained topo-I). For each extract used a number of sera of known specificity (and of relative specificity to the particular extract), were included on the gel as positive controls. Each serum sample was tested a number of times: with each extract, and alongside sera of various specificities, until the resulting precipitin line was identified.

2.4.4: Immunoprecipitation of proteins by SDS polyacrylamide gel electrophoresis

The method used was based on the original method described by Craft, Mimori and Olsen ('88) (162). Serum was incubated with Sepharose beads to which protein-A was bound (Protein-A Sepharose CL). IgG present in the serum bound to the protein-A via the Fc fragment. The beads were washed free of any unbound material and re-incubated with a radiolabelled human cell extract. Any unbound material again was washed away. Bound antigens were then removed from the Sepharose protein-A, reduced and denatured by boiling in standard Llaemmli sample buffer. Constituent polypeptides were then separated by vertical SDS-PAGE followed by detection by autoradiography and identification according to banding pattern.

Radiolabelling of K562 cells with ^{35}S-labelled L-methionine

Precautions appropriate to the nature and quantities of radiochemical used were observed throughout.

Three 600ml tissue culture flasks (Falcon brand, 3045) of K562 cells were grown to semiconfluency in RPMI +10%FCS. Four hundred ml of cell suspension (enough to provide approximately 80 million cells) was divided into 50ml centrifuge tubes and spun at 1000r.p.m. for 7 min. The supernatant was poured off and the pellets resuspended in 2-3ml serum-free methionine-free RPMI. The suspension was pooled into one tube and a sample was counted on a haemocytometer to calculate the number of cells. The volume was made up to 50ml and the tube agitated to fully resuspend and wash the cells. The cells were pelleted as before, the supernatant poured off and the pellet resuspended in 10ml methionine free + 5% d-FCS. The cells were recounted and the appropriate volumes were transferred into sterile 250ml flasks (20 million cells/flask), to which methionine free + 5% d-FCS was added to a final volume of 90ml. The final cell density was 2-2.5 million cells/ml. Flasks were placed in an incubator for 30min to deplete the cells of methionine. Approximately 160µl of ^{35}S-labelled L-methionine solution (6.7µM, 10mCi/ml NEN Biologicals, NEN Dupont, Stevenage, U.K.) was added to the flasks, enough to give a final activity of 20µCi/ml. The flasks contents were swirled gently to mix and the flasks were returned to the incubator for overnight incubation.

Preparation of the ^{35}S-radiolabelled K562 cell extract

When possible, during the preparation of the cell extract, materials being used were kept on ice.

After overnight incubation the cells were examined to check that they were still viable. If they appeared 'unhealthy' for any reason or dead the protocol was not continued any further and the cells were discarded. If the cells appeared satisfactory then the cell suspension was divided into two 50ml (Falcon) centrifuge tubes, and spun for 10min at 1000rpm. The supernatant was poured off and the cell pellet resuspended by brief vortex in ice cold Tris Buffered Saline (TBS) (10mM Tris.CL pH7.4, 150mM NaCl). The suspension was pooled into one tube and spun at 1000r.p.m for 10 min. The supernatant was poured off, and the pellet resuspended in 8ml of cell lysing Immunoprecipitation Buffer (IPP) (10mM Tris.CL pH 8.0, 500mM NaCl, 0.1% nonidet P-40) by brief vortex. The suspension was sonicated (Soniprep 150 MSE), with the sonicator set to mark (setting) 3, for 3 x 45 second blasts separated by 2 X 20 second intervals, after which the suspension was transferred to sterile 1.7ml Eppendorf tubes (1000µl/tube). The Eppendorf tubes were centrifuged in a Beckman GS-15R/F2402 at 12000 r.p.m, for 20min at 1°C to remove insoluble debris. The supernatant was then transferred to fresh Eppendorf tubes and stored at -70°C. The pellets were discarded.

Incubation of sera with protein Sepharose A beads

Known standard control sera and test serum samples were removed from –25°C storage and allowed to thaw at room temperature. Protein-A Sepharose CL-4B (Sigma chemical company Ltd.) was weighed out; enough for 2mg per serum sample. The Sepharose was prepared for use by removing the inactivating cyanogen bromide according to the manufacturers instructions. An appropriate volume of IPP was added to the Sepharose to reach a final concentration of 4mg/ml. The IPP was added a couple of ml at a time in order to ensure that all the Sepharose was transferred to a clean polypropylene tube (Falcon). The tube was tightly closed and put on end over rotation in a cold cabinet (4°C) for 1 hour for the Sepharose to hydrate. One point seven ml volume Eppendorf Safe-Twist tubes (Original Eppendorf, 22408) were labelled with a serum ID code, and 500µl of Sepharose suspension was added to each tube. Ten µl of serum were added to the respective tube containing the Sepharose suspension and securely closed. The tubes

were incubated overnight in the cold cabinet with end over rotation to charge the Sepharose with immunoglobulin.

Incubation of immunoglobulin-charged beads with S^{35}-radiolabelled cell extract

The following was carried out on ice wherever possible.

After incubation samples were subjected to a series of washes in order to remove unbound material. The tubes were pulse spun at 13000rpm for 10 seconds. The supernatant was removed from each Eppendorf tube with care taken not to disturb the pellet. Each pellet was resuspended in 500µl ice cold IPP buffer and then each tube vortexed briefly. This process was repeated until the tubes had been spun 4 times. After the 4^{th} spin the supernatant was removed and each pellet was resuspended in 400µl IPP buffer. The tubes were briefly vortexed to resuspend the pellet. One hundred µl (approximately 1-1.5 million cells) of the prepared radiolabelled cell extract were added to each Eppendorf tube. The tubes were resealed and incubated in a cold cabinet for 2 hours with end over rotation.

After incubation the samples were washed as follows: Tubes were pulse spun, and the supernatant removed as before. Five µl of ice-cold IPP buffer were added to each sample and resuspended by brief vortex. This process was repeated 3 times, so the tubes had been spun a total of 4 times. After the 4^{th} spin the supernatant was removed. Special care was taken to remove as much of the buffer as possible without disturbing the pellet. Pellets were resuspended in 40µl Llaemmli denaturing sample buffer (62.5mM Tris HCL pH 6.8, 10% glycerol, 5% B-mercaptoethanol, 0.0025% (w/v) bromophenol blue) by brief vortex. The tubes were spun briefly in the microfuge. The Eppendorf tubes (beads and sample) were stored at -70°C.

Assembly of apparatus for polyacrylamide gel electrophoresis

Plates were washed under running water, dried, and cleaned with ethanol. The gel apparatus (Hoefer SE 600 Series) was assembled according to the manufacturer instructions. Appropriate spacers were used for the required gel thickness. For the following, 1ml thick spacers, and 15 well, 1ml thick combs were used.

Producing and pouring the acrylamide gel

The lower, 10% acrylamide 'lower' gel (appendix III) was prepared, and poured between the plates, leaving a space of approximately 3cm from the top of the plates. The gel was overlaid with water saturated butan-2-ol (60ml butan-2-ol and 40ml ddH2O, shaken and allowed equilibrate) and left to polymerise. The upper 5% acrylamide gel was prepared (appendix III).

When the lower gel had set the top was rinsed free of butan-2-ol and any unpolymerised solution by rinsing between the tops of the plates with ddH_2O. Tissue was used to soak away residual drops. The upper gel was poured onto the top of the lower gel to approximately 0.5cm from the top of the plates. A 15 well comb was pushed into the top of the upper gel. The upper gel was left to set.

Sample preparation

The Safe-Twist Eppendorf tubes containing the samples (prepared in the method entitled "Incubation of immunoglobulin charged-beads with S^{35}-radiolabelled cell extract") were plunged into a beaker of boiling water for 5 min, ensuring that the neck of the tubes remained above the surface of the water.

Gel loading and electrophoresis

After the gel had set the comb was removed. The top of the gel was rinsed thoroughly with ddH_2O to remove any excess unpolymerised solution. The wells were then drained of water, using tissue to absorb the last drops, and then filled with running buffer (25mM Trizma base, 190mM glycine, 0.1% (w/v) SDS) Twenty µl of each prepared sample was underlayed into each of the allocated wells. Ten µl of high molecular weight colour marker were loaded into one well. The apparatus was fully assembled and attached to a power pack according to the manufacturer instructions. The gels were run at 30mAmps constant current until the bromophenol blue tracer dye (contained in the denaturing sample buffer) had moved through the 5% upper gel and into the 10% lower gel, when it was increased to 60mAmps for the duration. The run was terminated when the dye front approached the bottom of the plates.

Gel processing and drying

The apparatus was dismantled and the gel plates carefully prised apart. A scalpel blade was used to carefully cut away the upper gel, which was discarded. The lower portion of the gels were soaked in 0.5M sodium salicylate for 15 min, to enhance, followed by fixing in a fixative solution (methanol: water: glacial acetic acid, 4.5:4.5:1) for at least 0.5 hours. Gels were then placed onto a piece of blotting paper which had been cut to a size approximately $1cm^2$ larger to ease handling. A piece of cellophane (Cellophane membrane backing, BioRad Laboratories) about the same size as the blotting paper was placed over the exposed side of the gel. The Gel 'sandwich' was then placed onto the gel dryer (Rapidry gel dryer ATTA Electrophoresis) at $70^{O}C$ for 1.5 hours.

Autoradiography

The gel was exposed to photographic film (e.g. XASR-1 or XAR-2 from Kodak) and left for 1-5 weeks. The film was developed and the lanes labelled.

2.4.5: Immunoblotting

Immunoblotting or, *Western Blotting,* is used to identify specific antigens recognised by antibodies, according to their apparent molecular weights. The prepared antigen source is solubilised, separated by SDS-PAGE, and then electrophoretically transferred to a nitrocellulose membrane. Following this, the membrane is cut into strips, which are incubated firstly in a protein rich solution to block remaining (non-specific) binding sites, and then with a diluted sample of serum. Autoantibodies that recognise linear epitopes bind to the denatured antigens. The strips are then incubated with an alkaline phosphatase-conjugated anti-human immunoglobulin, followed by incubation with the appropriate alkaline phosphatase substrates, which results in 'coloured' product where conjugate has bound. The consequent bands are indicative of the molecular weights of the antigenic subunits recognised by autoantibodies in the sample serum.

Immunoblotting was carried out according to the method described by McHugh, James and Maddison ('88) (163), with some modification.

Antigen preparation for immunoblotting – unlabelled K562 cell extract

A nuclear sonicate of K562 cells was prepared. To all buffers 1mM phenylmethylsulphonyl fluoride, 10mM idoacetamide, and 10mM EDTA were added. K562 cells were grown to semi-confluency. Cells were washed twice in 0.01M Phosphate buffered with 0.15M saline (PBS) (pH7.4). Cells were then solubilized at a concentration of $5X10^7$ cells/ml in PBS containing 0.5% Triton X-100 for 30 min on ice. The tube was then spun at 5000g for 15 min at 4°C to pellet the nuclei. The supernatant was retained as the cytoplasmic fraction. The nuclei were resuspended in 1mM $NaPO_4$, pH7.0, and sonicated at least 10 times for 10sec until all the nuclei had disintegrated (as determined by light microscopy). The insoluble material was removed by centrifugation at 500g for 15 min at 4°C and the supernatant retained as the soluble nuclear fraction. One ml aliquots of the nuclear extract was pipetted into Eppendorfs and stored at –80°C until use.

SDS PAGE of (unlabelled) K562 cell extract for immunoblotting and electrophoretic transfer

The K562 cell sonicate (antigen source) was separated on a 12.5% polyacrylamide gel (according to the method entitled "gel loading and electrophoresis") with minor modification as follows; A comb with one large well and one small well was used. 200-300µl of sample, (which had been prepared according to the method "*sample preparation*"), was added to the large well and 15µl of molecular weight marker were added to the small well.

Electrophoretic Transfer onto nitrocellulose membranes

Transfer buffer (20mM Trizma base, 150mM glycine, 20% (v/v) methanol, 0.1% SDS) was freshly prepared using a 10x concentrate of Transfer Buffer Solution (200mM Trizma base, 150mM glycine). One hour prior to transfer, sheets of nitrocellulose membrane (BioRad laboratories), (cut to slightly larger than gel size), were placed in a container of ddH_2O to soak and subsequently washed in two changes of water and two changes of transfer buffer.

After SDS-PAGE, gels were carefully removed from the glass plates, and the stacking gel trimmed away with a scalpel blade. Gels were then placed into the transfer buffer and left for 10min to equilibrate. Nitrocellulose membrane, blotting paper, and fiber

pads, which had been cut to the dimensions of the gel, were also put into the transfer buffer to soak. The gel cassettes were assembled, whilst fully submerged in the transfer buffer, as follows; one fiber pad, two sheets of blotting paper, one SDS-polyacrylamide gel, one nitrocellulose membrane, two sheets of blotting paper, one fiber pad. To ensure the correct direction of movement of proteins (i.e. from the gel onto the nitrocellulose membrane), care was taken to check that the cassettes were assembled in such a way that the membrane was closest to the anode (+) side of the cassette and the gel was closest to the cathode (-) side of the cassette. Air bubbles caught between the gel and the membrane prevent a clean contact thus result in poor transfer. Any air bubbles were removed by gently rolling them out with a glass tube. Cassettes were firmly closed with the fitted clasp.

The gel sandwich was inserted into the running tank, which was then completely filled with transfer buffer. The cooling coil, which was attached to a cold water supply, was inserted into the vacant slot between the cassettes and a stir bar, ('flea'), was dropped into the tank, which helped to maintain uniform conductivity and temperature during electrophoretic transfer. The lid was pressed onto the top of the apparatus and the cables plugged into the power supply. The transfer was run for 2 hours at 250mA constant current. After this time the apparatus was partially dissembled so that a corner of the membrane could be checked to see that transfer of the coloured molecular weight markers had occurred. If they hadn't, then the transfer was incomplete, and the apparatus was re-assembled and run for a while longer.

Once the transfer was complete, the apparatus was fully dissembled. The gels were discarded and the nitrocellulose membranes were rinsed in ddH_2O. The membranes were then stained with Ponceau S to check for protein content. This was done by immersing the membrane in Ponceau S solution (0.2% Ponceau S, 1% (v/v) acetic acid) and agitating on a moving platform, for 5min, at room temperature. The membranes were then de-stained by subsequent washings with ddH_2O over a 10 min period and then left to dry on a clean piece of blotting paper.

Immunoblotting

After staining with Ponceau S, the nitrocellulose membranes were orientated and a pencil line drawn about 5mm from the bottom edge. The membrane was cut into strips

approximately 5mm in width, and the strips numbered according to the order in which they were cut. Strips were placed (1/well) into a (20 well) blotting tray (BioRad laboratories) and blocked with blotto (5% skimmed milk powder in PBS) (2ml/well); this served to block non-specific protein-binding sites and was carried out for 2 hours on a moving platform.

The blotto was carefully poured off and another 3ml added to each well, followed by 30µl of sample or prototype sera to allocated wells and incubated, at room temperature, on the moving platform for a further 2 hours at room temperature. After this time the liquid was poured off and the strips subjected to 3, 10min washes with PBS.

Strips were then incubated with the second antibody (anti-human IgG/IgM developed in goat, conjugated to alkaline phosphatase; Sigma Chemical Co. Ltd.), which was added at a concentration of 1/1000 (2µl in 2ml blotto/well). The blotting tray was returned to the moving platform for a further 2 hours, after which the strips were washed as before, drained, and then covered with 2ml/well blotting buffer (0.1M tris, 0.1M NaCl, 5mM $MgCl_2$, pH9.6 with HCl).

Developing the nitrocellulose strips

One hundred ml of developing solution (50ml blotting buffer, 100µl BCIP and 100µl NBT) were placed into a clean flat glass dish. The substrates, 200µl 5-bromo-4-chloro-3-indolyl phosphate solution (BCIP; 50 mg/ml in dimethylformamide (DMF)), and 200µl nitroblue tetrazolium solution (NBT; 50mg/ml in 70% (v/v) DMF) were added and the dish was agitated to swirl and mix the contents. Strips were individually removed from the wells and placed into the developing solution. The prototype sera (positive and negative controls) helped to gauge when the optimal development, (satisfactory contrast between positive bands and negative background), of strips was achieved. When the strips of membrane reached this point they were taken out of the developing solution, rinsed in tap water and left on a clean sheet of blotting paper to dry.

2.4.6: T-cell proliferative responses to full length human topoisomerase I recombinant protein

The following procedure was carried out by Jean Whyte, and not by myself. However, the results from her work are relevant to the present study and have been included. The following is a description of the methods she employed in order to generate the results contained in this report.

Generation of full length recombinant topoisomerase I protein from a Baculovirus expression system

The purification and characterisation of the recombinant human topo I expressed in *Baculovirus* has been previously described (164). Briefly, insect (Sf9) cells were infected with a high titre virus stock. At 50h post infection the cells were harvested, lysed with triton X-100, and the nuclei isolated. The DNA was precipitated from the nuclei following lysis in high salt (1M NaCl), with a final concentration of 6% PEG 8000. The supernatant was subjected to successive chromatography and the final pooled fractions were dialysed and stored at -20°C. Purified topo-I migrated as a single band of 100kD using polyacrylamide gel electrophoresis. The purified protein was identified by sera containing anti-topo I autoantibodies on immunoblots, and was enzymatically active as determined by the ability to relax supercoiled DNA.

Proliferation of lymphocytes using topoisomerase I

All procedures were carried out in sterile conditions until the cells were harvested. Lymphocytes were isolated from whole blood as follows; 20ml of blood were mixed with an equal volume of room temperature PBS and carefully layered onto 15ml of lymphoprep™ (Nycomed Pharma AS, Norway). The tube was spun for 30 min at 450g. The buffy coat was collected and diluted up to 40mls with ambient temperature RPMI medium. Cells were harvested at 350G for 5 min and washed once in 25ml RPMI medium, resuspended in 10ml of medium and counted using trypan blue. Concentration was adjusted to 1 X 10^6 cells/ml.

Flat bottomed 96 well plates (Falcon 3072) containing the antigen, topo I, were prepared. All wells were prepared in quadruplicate. Anti-human CD3 antibody was used as a positive control, and the antigen was omitted for negative controls. Fifty µl of either

antigen in medium or medium alone were added to each of the designated wells followed by 100 µl of the cell suspension.

Plates were placed in the CO_2 incubator at 37°C, (5% CO_2, 95% humidity) for 7 days. Cells incubated with anti-CD3 antibodies were pulsed with 1uCi [^{3}H] thymidine and harvested on days 3,4,5,6, and 7. Cells incubated with topo-I were pulsed similarly and harvested on days 5,6, and 7. Filters were counted in a liquid scintillation counter. Proliferation was expressed as a stimulation index (SI; c.p.m. in the presence of antigen divided by c.p.m. in the absence of antigen).

2.5: STATISTICAL ANALYSES

Statistical analyses were performed by Chi-squared analysis of 2 x 2 contingency tables. In cases where N was less than 100 and/or any cell was less than 10, Yates correction was applied. When $N<20$, or $20<N<40$ *and* the smallest expected value was less than 5 Fishers exact test was used. Bonforroni correction was applied to data that had been subject to multiple testing and to results showing previously unreported associations. Where significant P values were identified ($p \leq 0.05$), odds ratio and confidence intervals were also calculated.

CHAPTERS 3 - 6

RESULTS

CHAPTER 3

MHC ASSOCIATIONS WITH AUTOIMMUNE RESPONSES TO TOPOISOMERASE I AND RNA POLYMERASE III

3.1: INTRODUCTION

At first glance there appears to be a relatively weak genetic contribution to the development of SSc. There is certainly a lack of consensus concerning the contribution of HLA class II alleles in SSc. Many centres worldwide have reported abnormal prevalence of particular HLA alleles associated with SSc, although as yet, there is no clear pattern. Alleles frequently reported to be associated with SSc in Caucasians include HLA-DR1 (80;165), DR3 (165-167) and DR5 (165;168). HLA-DR2 and HLA-DR4 have been implicated in Japanese studies (169). Different associations do tend to be observed in non-Caucasian populations, thus, the variability in reported HLA associations in SSc, may reflect ethnic differences in the groups studied. This suggests that the division of ethnic groups is important in the consideration of HLA (and autoantibody) associations in SSc. HLA DQB1 associations have mainly been reported in association with autoantibody subgroups of SSc and will be addressed in the relevant section later.

Genetic polymorphism of HLA class II molecules determines the chemical surface of the peptide-binding groove and is the principle determinant of the specificity and affinity of peptide binding and T-cell recognition. T-cell recognition of (auto) antigens would provide help for B cells in the development of an (auto) antibody response. The selective and differential binding capacity for certain peptides, due to HLA class II polymorphism, introduces a mechanism for restricting immune responses. T-cell proliferative assays have demonstrated that presentation of topoisomerase-I peptides can be restricted by HLA class II alleles (46). This supports the hypothesis that T cell response to topo-I is not associated with disease but restricted by HLA class II alleles. Differential binding affinities in the peptide-binding groove could therefore restrict the production of autoantibodies, and it is this concept, which forms the basis of association between certain HLA class II alleles and autoantibodies in patients with SSc.

HLA class II associations with SSc become stronger if autoantibody-defined subgroups of disease are studied.

Studies in HLA and ATA in SSc

HLA DRB5, is frequently associated with SSc and includes the alleles DRB1*1101-1104 and *1201-1202. HLA DRB1*1101-1104 is the most commonly reported allele associated with ATA and SSc in Caucasians (81). In Japanese however HLA DRB1*1502 is often reported (85;170). The HLA DQB1 allele often associated with ATA positive SSc patients is HLA DQB1*0301, not only in Caucasians (American) (84;171;171) and American Blacks (84), but also American Choctaws (31) and Japanese, in which an association with DQB1*0601 is also reported (85). Fanning *et al.* reported a decrease of HLA-DQB1*05 in ATA positive patients in comparison to ATA negative patients, but not an increase in the previously reported allele HLA DQB1*0301 (64).

HLA associations have been supported by T-cell proliferation studies. A report by Kuwana *et al.* showed that in vitro T cell proliferation to recombinant topoisomerase-I was mostly restricted by HLA-DRB1, specifically the 1501/2, 1101/3/4 and 0701 alleles in Caucasoids (172).

It has been proposed that specific amino acid residues at certain positions common to particular HLA-DRB1 and HLA-DQB1 alleles have close associations with ATA responses. Reveille *et al.* ('92) reported that an HLA-DQB1 allele bearing the polar uncharged amino acid residue tyrosine at position 30 of the outermost domain of the DQB1 chain (HLA-DQB1: 30:Y) was found in all American Caucasian and American Black ATA SSc patients (84). This observation was not supported in an alternative American Caucasian population reported by Morel ('94) (171), or in Japanese (85). However the latter group suggested that a tyrosine residue at position 26 of the HLA DQB1 chain (HLA-DQB1: 26:Y) and the amino acid sequence FLEDR from position 67 to 71 of HLA-DRB1, (HLA-DRB1: ^{67}FLEDR^{71}) was important in ATA production (85). HLA-DRB1: ^{67}FLEDR^{71} was also reported to be of increased frequency in a UK Caucasian population in ATA SSc patients (64).

Another sequence thought to be significant in the production of ATA is HLA-DQB1 ^{71}TRAELDT^{77,} present on all HLA-DQB1*06, (except *0606), and *03 alleles, and is reported to have been increased in American and UK Caucasians (64;84;171).

Studies in anti-RNA polymerase antibodies and HLA

To date, there is only one publication regarding HLA associations with anti-RNA polymerase (ARA) autoantibodies (64). In the mentioned study, two autoantibody groups have been considered: ARA I/II/III and ARA I/III. When allele frequencies of the two groups were analysed, in comparison to each other or, combined and compared to the remaining ARA negative group, no significant associations were identified. When the groups were compared independently to their respective negative groups a significant increase in HLA-DQB1*0201 was reported in the ARA I/II/III group. This allele is linked with DRB1*0301 which was observed to be raised but did not reach significance (64).

Mutual exclusivity of autoantibodies

Previous studies carried out at Bath have identified three main groups of ARA sera, ARA I/III, ARA I/II/III and ATA/ARA II (+/- other autoantibody), which are considered to be mutually exclusive (173). Mutual exclusivity of ARA III and ATA groups are generally supported by other studies (64;85). A difference in HLA associations between mutually exclusive groups would support the hypothesis in this study regarding the role of HLA in autoantibody production. One report (which also considers the mostly mutually exclusive group ACA) did not find any striking association of HLA that could explain the observed mutual exclusivity of autoantibody groups (64). However, a crucial factor in this study was the consideration that ATA and ARA were entirely mutually exclusive, which is contrary to reports from Bath (173). Thus for HLA associations to be found it is necessary to identify those autoantibody groups which are truly mutually exclusive.

This chapter explores HLA class II associations with ATA and ARA III in SSc.

3.2: CHAPTER AIMS

The overall objective of the work detailed in this chapter was to identify any HLA-DRB1 and HLA-DQB1 characteristics that may distinguish autoantibody subgroups (ATA and ARA III), and their mutual exclusivity in SSc. This was investigated by means of the aims set out below:

I. To identify any associations of HLA DRB1 and/or DQB1 alleles, (at the allele level or with previously reported amino acids at particular loci), with autoantibody subgroup, in an ATA positive and an ARA III positive SSc population.

II. To investigate the contribution of HLA towards ATA and ARA III mutual exclusivity.

III. To investigate whether HLA class II alleles determine an individuals' ability to mount a mononuclear cell proliferative response to topoisomerase-I.

3.3: MATERIALS AND METHODS

Patients and controls

Two hundred and thirty two patients fulfilled the established criteria for SSc (156), and were accepted into the study. All patients were Caucasian.

Five healthy individuals with no clinical evidence of an autoimmune disorder, (independent from the main cohort of normal controls detailed below), and three patients with SLE, were included in the T cell proliferation study.

The 'normal control' population

Blood samples, for DNA isolation, were obtained from 250 donors attending the Southwest region blood transfusion centre. DNA was isolated from these samples.

Detection of autoantibodies

Indirect immunofluorescence (method 2.4.2) was used to screen all patients for anti-cytoplasmic antibody, antinuclear antibody (ANA) and antinucleolar antibody (ANoA) reactivity's. Autoantibody specificities of ANA and ANoA positive sera were identified by ^{35}S-immunoprecipitation (method 2.4.4). Where deemed necessary results were confirmed by Ouchterlony double immunodiffusion (method 2.4.3) (with the exception of ARA). For each method, comparisons were made with prototype reference sera.

HLA-DRB1 and HLA-DQB1 typing

HLA-DRB1 and DQB1 alleles were identified by PCR-SSP (method 2.2.2). HLA-DRB1 and DQB1 typing was performed on all the samples.

T cell proliferation responses to recombinant topoisomerase-I

T cell proliferation assays to recombinant topoisomerase-I were carried out according to method 2.4.6

Statistical Analysis

Statistical analysis was performed by X^2 analysis of 2 x 2 contingency tables with correction where appropriate, according to that described in the main materials and methods, 2.5. The data in table 3.1 has also been analysed using the relative

predispositional effects (RPEs) (174) method to identify allele association sequentially according to individual allele strength.

3.4: RESULTS

3.4.1: Serological profile of patients

Of the 232 SSc patients accepted into the study, 37 (16%) were ATA positive. Twenty-two patients had autoantibodies to topoisomerase-I only. Ten patients were ATA and ARA II positive: (ATA + ARA II: 8, ATA + ARA II + anti-Ro: 1, ATA + ARA II + Jo-I: 1). Four patients had ATA and anti-Ro antibodies: (ATA + anti-Ro: 2, ATA + anti-Ro + anti-Jo-I: 1, ATA + anti-Ro + anti-La: 1). One patient was ATA and anti-U1RNP (+/-sm) positive.

Twenty-Seven (11.5%) patients were ARA III positive (ARA I/III: 6, ARA I/II/III: 20, ARA III: 1).

3.4.2: HLA-DRB1 and DQB1 phenotype frequencies in ATA positive patients

HLA-DRB1 and DQB1 phenotype frequencies in ATA positive patients

HLA-DRB1 and DQB1 phenotype frequencies of ATA positive SSc patients were compared with normal controls and ATA negative SSc patients. Analysis was also carried out within the ATA group with regard to the presence or absence of ARA II.

DNA was available from 28 of the 37 ATA positive patients (ATA: 17, ATA + ARA II phosphorylated (ARA II^{o}), + Ro: 1, ATA + ARA II^{o} + Jo-1: 1, ATA + ARA II^{o}: 4, ATA + ARA II^{o} + ARA II^{A}: 2, ATA + Ro: 1, ATA + Ro + Jo-1: 1, ATA + Ro + La: 0 and ATA + U1RNP +/-Sm: 1), on which HLA-DRB1 and DQB1 typing was carried out.

ATA positive patients vs. normal controls

Comparison of HLA-DRB1 phenotype frequencies between ATA positive patients and controls showed an increase in HLA DR5 in ATA positive patients (9/28 patients *vs.* 48/250 controls, p=0.1, table 3.1) although this was not significant (Table 3.1). However, when the DR5 subgroups (DRB1*11 and 12) were considered, a significant increase in DRB1*11 was identified in patients ($p < 0.05$ *vs.* controls), whilst the

DRB1*12 constituent of DR5 showed a negligible change in frequency compared to the controls (0/28 *vs.* 7/250, p=0.36) Although there was an increase observed in DRB1*0101-0103, (10/28 patients *vs.* 53/250 controls, p<0.1) and a reduction in HLA DRB1*04 (6/28 patients *vs.* 88/250 controls, p<0.1) these differences from the control population did not reach significance.

There were no significant differences in the frequency of HLA-DQB1 alleles in ATA positive patients compared to controls. HLA-DQB1*0501 and 0602 were reduced in the ATA positive group to some degree (9/28 *vs.* 52/250, p=0.16; 10/28 *vs.* 60/250, p=0.17 respectively).

ATA positive patients vs. ATA negative patients

A comparison of the phenotype frequency of ATA positive SSc patients with the remaining SSc patients, (ATA negative), showed significant differences (Table 3.1). HLA DR5 was significantly increased in ATA positive patients compared to ATA negative patients (9/28 ATA+ *vs.* 17/153 ATA-, p<0.005). When the patients comprising DR5 were considered in their subgroups the significant difference was present in the DRB1*11 constituent, and not DRB1*12 (9/28 *vs.* 14/153 p<0.001). HLA-DRB1*0301-0303 was significantly decreased in ATA positive patients (4/28 ATA+ *vs.* 58/153 ATA-, p<0.01).

Significant differences were also observed in HLA-DQB1 frequencies between these two groups. HLA-DQB1*0602 was significantly increased in ATA positive patients (10/28 ATA+ *vs.* 25/156 ATA-, p<0.02), as was HLA DQB1*0301 (13/28 ATA+ *vs.* 42/156 ATA-, p<0.04). HLA-DQB1*0201 was reduced in frequency in ATA positive patients compared to ATA negative patients, however this difference did not reach significance (8/28 ATA+ *vs.* 42/156 ATA-, p<0.1).

A relative predispositional effects analysis (174) was also carried out to sequentially compare allele frequencies in ATA positive patients and in the controls, to determine their predispositional, protective, or neutral effects relative to each other. However, for HLA-DRB1 and DQB1, no allele could be identified as having a significantly greater effect than any other allele, in this population, by this analysis (tables 3.2 and 3.3).

3.4.3: HLA DRB1 and DQB1 phenotype frequencies in ARA III positive patients

HLA-DRB1 and DQB1 phenotype frequencies of ARA III positive SSc patients were compared with normal controls and ARA III negative SSc patients (Table 3.4). Analysis was also carried out within the ARA III group with regard to the presence or absence of ARA II.

DNA was available from 16 of the ARA III positive patients (ARA I/III: 3, ARA I/II/III: 13, ARA III: 0) on which HLA-DRB1 and DQB1 typing was carried out.

ARA III positive patients vs. normal controls

There were no significant differences in phenotype frequencies when ARA III positive patients were compared with controls (Table 3.4).

ARA III positive patients vs. ARA III negative patients

No significant differences in HLA-DRB1 or DQB1* frequencies were observed between the ARA III positive and ARA III negative population (Table 3.4).

3.4.4: Analyses of reported HLA-DQB1 amino acid associations with SSc

HLA-DQB1 amino acid positions in ATA SSc patients

HLA-DQB1: 30:Y

Of the HLA-DQB1 alleles investigated in this study, 5 had a tyrosine residue at position 30 (HLA-DQB1*0601, 0602, 0301/4, 0302 and 0303). The possession of at least one HLA-DQB1: 30:Y allele was significantly increased in ATA positive patients compared to ATA negative patients (25/28 ATA+ *vs.* 101/156 ATA-, $p<0.025$) and was not significantly increased in ATA positive patients compared to normal controls ($p=0.13$).

HLA-DQB1 ^{71}TRAELDT^{77}

HLA-DQB1 ^{71}TRAELDT^{77}.was increased in ATA positive patients compared to controls although this observation did not reach significance ($p<0.1$). When ATA positive patients were compared to ATA negative patients the occurrence of HLA-DQB1

$_{71}$TRAELDT$_{77}$ was significantly increased in the ATA positive group (27/28 *vs.* 113/156, $p<0.025$).

DQB1: 26:Y

The possession of at least one HLA-DQB1: 26:Y positive allele was significantly increased in ATA positive patients compared to ATA negative patients (13/28 ATA+ *vs.* 44/156 ATA-, p=0.05). There were no significant differences in frequency observed at this locus when ATA positive patients were compared to controls.

HLA-DQB1 amino acid positions in ARA III SSc patients

HLA-DQB1: 30:Y

There were no significant differences in the frequency of HLA-DQB1: 30:Y in ARA III positive patients compared to controls or compared with ARA III negative patients.

HLA-DQB1 ^{71}TRAELDT^{77}

A reduction in the phenotype frequency of HLA-DQB1: ^{71}TRAELDT^{77} was observed in ARA III positive patients compared to controls, although this failed to reach significance (10/16 ARA III+ vs. 210/250 controls, p=0.06). No significant differences were observed when the phenotype frequencies of alleles positive for this locus were compared between ARA III positive patients and ARA III negative patients.

DQB1: 26:Y

No significant differences were observed in the occurrence of alleles possessing HLA: 26:Y in ARA III patients compared to controls or when compared to ARA III negative patients.

3.4.5: Heterogeneity of autoantibody groups

It is possible that the current autoantibody grouping may still represent a heterogeneous group, which could be further defined by HLA. Therefore consideration of HLA 'within' an autoantibody group may identify subgroups. Identification of HLA

associations within an autoantibody group (with regard to other autoantibodies present) would identify an autoantibody subgroup which may in turn be associated with a particular (more defined) clinical profile.

HLA phenotype frequencies were considered by comparisons within the ATA group with regard to ARA II (thus; ATA/ARA II+ *vs.* ATA/ARA II negative) and the ARA III group also with regard to ARA II, (ARA I/II/III *vs.* ARA I/III).

Further division of the ATA group

Twenty-seven patients were ATA positive /ARA II negative of which 20 were HLA typed, and 10 patients were ATA/ARA II positive of which eight were HLA typed. Fishers exact test was used due to the small number of individuals and the resulting p values identified a non-significant reduction of HLA-DRB1*1101-1104 in the ATA/ARA II negative group (ATA/ARA II negative; 4/20 *vs.* 5/8 ATA/ARA II+, p=0.06) (Table 3.6).

Further division of the ARA III group

Twenty patients were ARA I/II/III positive of which 13 were HLA typed. Six patients were ARA I/III positive, of which 3 were HLA typed and one patient was ARA III only positive and DNA was not available from this patient. Therefore, statistical analysis within this group was fairly meaningless due to the sample size. However, using Fishers exact test HLA-DRB1*0701-0702 appeared to be increased in the ARA I/III only group compared to ARA I/II/III group (ARA I/II/III 2/13 vs. 2/3 ARA I/III only positive patients, p=0.07) (Table 3.7).

3.4.6: Mutual exclusivity of autoantibodies

As mentioned earlier, specific autoantibodies are considered mutually exclusive, and give rise to recognised serological 'autoantibody' groups. An HLA association identified in one autoantibody group compared to a negative association in another would help to explain, and support, the observed mutual exclusivity of autoantibody groups.

ATA positive patients vs. ARA III positive patients

The comparison of HLA-DRB1 and DQB1 phenotype frequencies between ATA and ARA III positive patients did not show any significant differences. HLA DRB1*1501-1502 was raised in ATA positive patients compared to ARA III positive patients (2/16 ARA III *vs.* 10/28 ATA, p = 0.09, (p=0.18 corrected)), and DRB1*0301-0303 was reduced in ATA positive patients compared to ARA III positive patients (6/16 ARA III vs. 4/28 ATA, p = 0.07, (p corrected = 0.16)). The HLA-DQB1 alleles 0201 and 0602 showed the greatest differences in frequencies when the groups were compared, however, these results also, did not reach significance (p=0.15 ($p^{corrected}$=0.20) and p=0.09 ($p^{corrected}$=0.16) respectively). HLA-DRB1*1501-1502 and 0301-0303 are in linkage disequilibrium with HLA-DQB1*0201 and 0602 respectively. These results may become significant if the groups were larger.

HLA-DQB1: 30:Y

The possession of at least one allele possessing HLA-DQB1: 30:Y was significantly increased in ATA positive patients when compared to ARA III positive patients (25/28 ATA + *vs.* 9/16 ARA III +, $p^{corrected}<0.05$).

HLA-DQB1: ^{71TRAELDT77}

The possession of at least one allele possessing HLA-DQB1: ^{71TRAELDT77} was significantly increased in ATA positive patients when compared to ARA III positive patients (27/28 ATA + *vs.* 10/16 ARA III +, $p^{corrected}$ =0.01).

HLA-DQB1: 26:Y

When the frequency of the possession of at least one HLA-DQB1: 26:Y positive allele was compared between ATA positive patients and ARA III positive patients, no significant differences were observed.

3.4.7: Investigation of T cell proliferative responses to recombinant topoisomerase I

Patients and methods

Four patients with SSc, three patients with SLE and five healthy individuals with no clinical evidence of an autoimmune disorder were included in the T cell proliferation study. For all the individuals, T cell proliferation assays were performed. Autoantibody analysis and MHC class II typing were also carried out.

T cell proliferative responses to recombinant topoisomerase I

A T cell response to anti-CD3 antibodies was observed in all subjects. The maximum stimulation was observed on day 4 over the range of concentrations used. The SI in all subjects was greater than 8 (data not shown).

A proliferative peripheral blood mononuclear cell (PBMC) response to topo I was observed in a dose dependent manner in both a patient with anti-topo I antibodies and in a normal control but not in a patient with anti-centromere antibodies (Figure 3.1). In further experiments a concentration of 20 μg/ml of topo I was used to measure the proliferative response of a range of anti-topo I antibody positive and negative patients and controls.

A proliferative PBMC response to soluble topo I was present in 9/16 individuals (Table 3.8). A positive response was found in 3/3 ATA positive patients with SSc, one of five SSc patients without ATA, none of the three SLE patients and all of five normal controls. The greatest proliferative response (SI = 10) was observed in a normal control. In all subjects with a SI greater than three the optimum response was seen on day 7.

HLA class II type and T cell response to topoisomerase I

The above results suggest that a proliferative response to topo I may not be associated exclusively with disease status or the presence of anti-topo I antibodies. Therefore, it was considered whether there might be an association between the response, to topo I, and the presence of particular HLA-DRB1 and HLA-DQB1 alleles alone, or in combination.

The HLA-DRB1 and HLA-DQB1 status of the individuals studied for a proliferative response to topo I is shown in Table 3.8. An increase in HLA-DRB1*1101-1104 was observed in responders compared to non-responders (33% *vs.* 0%). Conversely, an increase in HLA-DR4 was observed in the non-responders (71.4% *vs.* 22% in responders). HLA-DQB1*0302 was slightly increased in the responders compared to the non-responders (16.7% *vs.* 7.1%). There was an increase in the HLA-DQB1*0201 allele in the non-responders (71.4% *vs.* 22% in responders). However, none of these differences were significant after correction for the number of variables tested.

As mentioned previously, Kuwana *et al.* found that all SSc patients and healthy controls with any of HLA-DRB1*1501,2 (DR15), DRB1*1101,3,4 (DR11), or DRB1*07 (DR7) alleles exhibited T cell responses to topo I recombinant fusion proteins (172). The results here are in general agreement in that seven of the nine responders had at least one of these HLA-DRB1 alleles. However, two of seven non-responders also had HLA-DRB1 *1501 alleles.

HLA-DQB1: 30:Y and T cell response

It was investigated whether the ability to respond to topo I was determined by HLA-DQB1: 30:Y status. All but one responder had at least one HLA-DQB1: 30:Y allele. However, five of seven non-responders also had at least one HLA-DQB1: 30:Y allele. Of interest, HLA-DQB1: 30:Y homozygosity was present in five of nine responders and in nought of the seven non-responders ($p < 0.03$) (Table 3.8). One interpretation of this finding is that a protective factor on a non-HLA-DQB1: 30:Y allele may help prohibit a response to topo I.

There may be interaction between HLA-DRB1 and HLA-DQB1 alleles that determines the ability to mount a T cell response to topo-I as has been suggested by Kuwana *et al.* (85). We considered whether the possession of an HLA-DRB1 allele or common sequence shared by HLA-DRB1 alleles might confer susceptibility, and a non-HLA-DQB1: 30:Y allele may be protective. However, there was no combination of HLA-DRB1 alleles with HLA-DQB1: 30:Y alleles observed in our responders or non-responders that supported this model (which may be due to the small numbers of individuals studied as some initial trends were apparent).

The possibility that HLA-DQB1: 30:Y alleles may confer susceptibility whereas HLA-DRB1 alleles such as HLA DR4 may be protective was then considered. Furthermore, it was considered whether homozygosity for HLA-DQB1: 30:Y alleles may override the 'protective' effect of HLA-DR4. This model was applied to the present data and that of Kuwana *et al.* (172) (Table 3.9). The model could explain responsiveness to topo I in 15/16 of the patients detailed here and in 49/55 patients described by Kuwana *et al.* (172).

Table 3.1: Comparison of HLA-DRB1 and DQB1 phenotype frequencies of ATA positive patients with ATA negative patients and normal controls

Allele		Controls		ATA positive		ATA negative	
HLA DRB1*		(n=250)	(%)	(n=28)	(%)	(n=153)	(%)
0101-0103	(DR1)	53	(21.2)	10	(35.7)	37	(24.2)
1501-1502	(DR2)	65	(26.0)	10	(35.7)	28	(18.3)
1601-1602	(DR2)	6	(2.4)	0	(0.0)	2	(1.3)
Sum DR2		71	(28.4)	10	(35.7)	30	(19.6)
0301-0303	(DR3)	53	(21.2)	4*[4]	(14.3)	58*[4]	(37.9)
0401-0411	(DR4)	88	(35.2)	6	(21.4)	51	(33.3)
1101-1104	(DR5)	41*[1]	(16.4)	9*[1, *3]	(32.1)	14*[3]	(9.2)
1201-1202	(DR5)	7	(2.8)	0	(0.0)	3	(2.0)
Sum DR5		46	(19.2)	9*[2]	(32.1)	17*[2]	(11.1)
1301-1305	(DR6)	54	(21.5)	4	(14.3)	20	(13.1)
1401-1410	(DR6)	5	(2.0)	0	(0.0)	5	(3.3)
0701-0702	(DR7)	63	(25.2)	5	(17.9)	30	(19.6)
0801-0805	(DR8)	13	(5.2)	1	(3.6)	10	(6.5)
0901	(DR9)	4	(1.6)	1	(3.6)	2	(1.3)
1001	(DR10)	2	(0.8)	0	(0.0)	2	(1.3)
HLA DQB1*		(n=250)		(n=28)		(n=156)	
0201	(DQ2)	88	(35.2)	8	(28.6)	75	(48.1)
0401	(DQ4)	0	(0.0)	0	(0.0)	0	(0.0)
0402	(DQ4)	10	(4.0)	0	(0.0)	8	(5.1)
0501	(DQ5)	52	(20.8)	9	(32.1)	32	(20.5)
0502	(DQ5)	9	(3.6)	0	(0.0)	1	(0.6)
0503	(DQ5)	5	(2.0)	1	(3.6)	4	(2.6)
0601	(DQ6)	3	(1.2)	0	(0.0)	2	(1.3)
0602	(DQ6)	60	(24.0)	10*[5]	(35.7)	25*[5]	(16.0)
0603/8	(DQ6)	27	(10.8)	2	(7.1)	11	(7.1)
0604	(DQ6)	28	(11.2)	2	(7.1)	10	(6.4)
0301	(DQ7)	91	(36.4)	13*[6]	(46.4)	42*[6]	(26.9)
0302	(DQ7)	51	(20.4)	5	(17.9)	42	(26.9)
0303	(DQ7)	31	(12.4)	4	(14.3)	12	(7.7)

Comparisons of HLA-DRB1* and HLA-DQB1* phenotype frequencies in ATA positive SSc patients compared to ATA negative patients and normal controls. p values with OR(CI) are: *[1]<0.05: 0.4(0.1-0.9), *[2]<0.005: 3.7(1.4-9.6), *[3]<0.001:4.7(1.7-12.3), *[4]<0.01: 0.27(0.09-0.82), *[5]<0.02: 2.9(1.2-7.0), *[6]<0.04: 2.3(1.0-5.4) when compared.

Table 3.2: Relative predispositional effects analysis of HLA-DRB1 alleles in ATA positive SSc patients

	All alleles (n =56)		
HLA-DRB1* allele	**Observed**	**Expected**	X^2
0101-0103	10	6.3	2.2
1501-1502	11	8.2	1
1601-1602	0	0.7	0.7
0301-0303	5	6.7	0.4
0401-0411	7	10.6	1.2
1101-1104	11	4.8	8
1201-1202	0	0.8	0.8
1301-1305	5	6.4	0.3
1401-1410	0	0.6	0.6
0701-0702	5	8.4	1.4
0801-0805	1	1.7	0.3
0901	1	0.5	0.5
1001	0	0.3	0.3

$X^2 = 17.7$

Degrees of freedom = 12

P = 0.1 (not significant)

The table shows the predispositional effects analysis for HLA-DRB1 alleles in ATA positive SSc patients. Using this analysis, no particular allele could be identified as having a significantly greater effect in this group of patients than the other alleles.

Table 3.3: Relative predispositional effects analysis of HLA-DQB1 alleles in ATA positive SSc patients

	All alleles (n =56)		
HLA-DQB1* allele	**Observed**	**Expected**	X^2
0201	7	11.5	1.8
0402	0	1.3	1.3
0501	9	6.2	1.3
0502	0	1.0	1.0
0503	1	0.6	0.3
0601	0	0.3	0.3
0602	12	7.6	2.5
0603/8	2	3.2	0.45
0604	2	3.2	0.45
0301	14	11.4	0.6
0302	5	6.0	0.2
0303	4	3.5	0.07

$X^2 = 10.27$

Degrees of freedom = 11

P = 0.5 (not significant)

The table shows the predispositional effects analysis for HLA-DQB1 alleles in ATA positive SSc patients. Using this analysis, no particular allele could be identified as having a significantly greater effect in this group of patients than the other alleles.

Table 3.4: HLA-DRB1 and DQB1 allele phenotype frequencies of ARA III positive and negative patients and normal controls

Allele		Controls		ARA III positive		ARA III negative	
HLA DRB1*		(n=250)	(%)	(n=16)	(%)	(n= 165)	(%)
0101-0103	(DR1)	53	(21.2)	6	(37.5)	49	(29.7)
1501-1502	(DR2)	65	(26.0)	2	(12.5)	36	(21.8)
1601-1602	(DR2)	6	(2.4)	0	(0.0)	2	(1.2)
Sum DR2		71	(28.4)	2	(12.5)	38	(23.0)
0301-0303	(DR3)	53	(21.2)	6	(37.5)	60	(36.4)
0401-0411	(DR4)	88	(35.2)	5	(31.3)	59	(35.8)
1101-1104	(DR5)	41	(16.4)	2	(12.5)	16	(9.7)
1201-1202	(DR5)	7	(2.8)	0	(0.0)	3	(1.8)
Sum DR5		46	(19.2)	2	(12.5)	19	(11.5)
1301-1305	(DR6)	54	(21.5)	1	(6.3)	25	(15.2)
1401-1410	(DR6)	5	(2.0)	1	(6.25)	1	(0.6)
0701-0702	(DR7)	63	(25.2)	2	(12.5)	31	(18.8)
0801-0805	(DR8)	13	(5.2)	1	(6.3)	9	(5.5)
0901	(DR9)	4	(1.6)	0	(0.0)	3	(1.8)
1001	(DR10)	2	(0.8)	0	(0.0)	2	(1.2)
HLA DQB1*		(n=250)		(n=16)		(n=169)	
0201	(DQ2)	88	(35.2)	8	(50.0)	76	(45.0)
0401	(DQ4)	0	(0.0)	0	(0.0)	0	(0.0)
0402	(DQ4)	10	(4.0)	1	(3.3)	7	(4.1)
0501	(DQ5)	52	(20.8)	4	(25.0)	55	(32.5)
0502	(DQ5)	9	(3.6)	0	(0.0)	1	(0.6)
0503	(DQ5)	5	(2.0)	1	(6.25)	4	(2.4)
0601	(DQ6)	3	(1.2)	0	(0.0)	1	(0.6)
0602	(DQ6)	60	(24.0)	2	(12.5)	33	(19.5)
0603/8	(DQ6)	27	(10.8)	1	(6.25)	12	(7.1)
0604	(DQ6)	28	(11.2)	0	(0.0)	12	(7.1)
0301	(DQ7)	91	(36.4)	4	(25.0)	51	(30.2)
0302	(DQ7)	51	(20.4)	4	(25.0)	43	(25.4)
0303	(DQ7)	31	(12.4)	1	(6.3)	14	(8.3)

Comparisons of allele phenotype frequencies of ARA III positive SSc patients with normal controls, and with ARA III negative patients showed no significant differences.

Table 3.5: Comparison of HLA-DRB1 and DQB1 phenotype frequencies of ARA III positive and ATA positive patients

Allele		ARA III positive		ATA positive	
HLA DRB1*		(n=16)	(%)	(n=28)	(%)
0101-0103	(DR1)	6	(37.5)	10	(35.7)
1501-1502	(DR2)	2	(12.5)	10	(35.7)
1601-1602	(DR2)	0	(0.0)	0	(0.0)
Sum DR2		2	(12.5)	10	(35.7)
0301-0303	(DR3)	6	(37.5)	4	(14.3)
0401-0411	(DR4)	5	(31.3)	6	(21.4)
1101-1104	(DR5)	2	(12.5)	9	(32.1)
1201-1202	(DR5)	0	(0.0)	0	(0.0)
Sum DR5		2	(12.5)	9	(32.1)
1301-1305	(DR6)	1	(6.3)	4	(14.3)
1401-1410	(DR6)	1	(6.3)	0	(0.0)
0701-0702	(DR7)	2	(12.5)	5	(17.9)
0801-0805	(DR8)	1	(6.3)	1	(3.6)
0901	(DR9)	0	(0.0)	1	(3.6)
1001	(DR10)	0	(0.0)	0	(0.0)
HLA DQB1*		(n=16)		(n=28)	
0201	(DQ2)	8	(50.0)	8	(28.6)
0401	(DQ4)	0	(0.0)	0	(0.0)
0402	(DQ4)	1	(3.3)	0	(0.0)
0501	(DQ5)	4	(25.0)	9	(32.1)
0502	(DQ5)	0	(0.0)	0	(0.0)
0503	(DQ5)	1	(6.3)	1	(3.6)
0601	(DQ6)	0	(0.0)	0	(0.0)
0602	(DQ6)	2	(12.5)	10	(35.7)
0603/8	(DQ6)	1	(6.25)	2	(7.1)
0604	(DQ6)	0	(0.0)	2	(7.1)
0301	(DQ7)	4	(25.0)	13	(46.4)
0302	(DQ7)	4	(25.0)	5	(17.9)
0303	(DQ7)	1	(6.3)	4	(14.3)

The comparison of HLA-DRB1* and DQB1 phenotype frequencies between ATA and ARA III positive patients showed no significant differences. However, an increase in HLA-DRB1 *1501-1502, and a reduction in DRB1*0301-0303, was observed in ATA positive patients. Variation in HLA-DQB1 phenotype frequencies were also observed in the HLA-DQB1*0201 and *0602 alleles.

Table 3.6: Comparison of HLA-DRB1 and DQB1 phenotype frequencies within the ATA positive group

Allele		ATA +/- other Aab.		ATA + ARA II	
HLA DRB1*		(n=20)	(%)	(n=8)	(%)
0101-0103	(DR1)	8	(40.0)	2	(25.0)
1501-1502	(DR2)	9	(45.0)	1	(12.5)
1601-1602	(DR2)	0	(0.0)	0	(0.0)
Sum DR2		9	(45.0)	1	(12.5)
0301-0303	(DR3)	1	(5.0)	1	(12.5)
0401-0411	(DR4)	5	(25.0)	1	(12.5)
1101-1104	(DR5)	4	(20.0)	5	(62.5)
1201-1202	(DR5)	0	(0.0)	0	(0.0)
Sum DR5		4	(20.0)	5	(62.5)
1301-1305	(DR6)	2	(10.0)	2	(25.0)
1401-1410	(DR6)	0	(0.0)	0	(0.0)
0701-0702	(DR7)	3	(15.0)	2	(25.0)
0801-0805	(DR8)	1	(5.0)	0	(0.0)
0901	(DR9)	0	(0.0)	1	(12.5)
1001	(DR10)	0	(0.0)	0	(0.0)
HLA DQB1*		(n=20)		(n=8)	
0201	(DQ2)	5	(25.0)	3	(37.5)
0401	(DQ4)	0	(0.0)	0	(0.0)
0402	(DQ4)	0	(0.0)	0	(0.0)
0501	(DQ5)	7	(35.0)	2	(25.0)
0502	(DQ5)	0	(0.0)	0	(0.0)
0503	(DQ5)	0	(0.0)	1	(12.5)
0601	(DQ6)	0	(0.0)	0	(0.0)
0602	(DQ6)	9	(45.0)	1	(12.5)
0603/8	(DQ6)	1	(5.0)	1	(12.5)
0604	(DQ6)	1	(5.0)	1	(12.5)
0301	(DQ7)	8	(40.0)	5	(62.5)
0302	(DQ7)	3	(15.0)	1	(12.5)
0303	(DQ7)	2	(10.0)	2	(25.0)

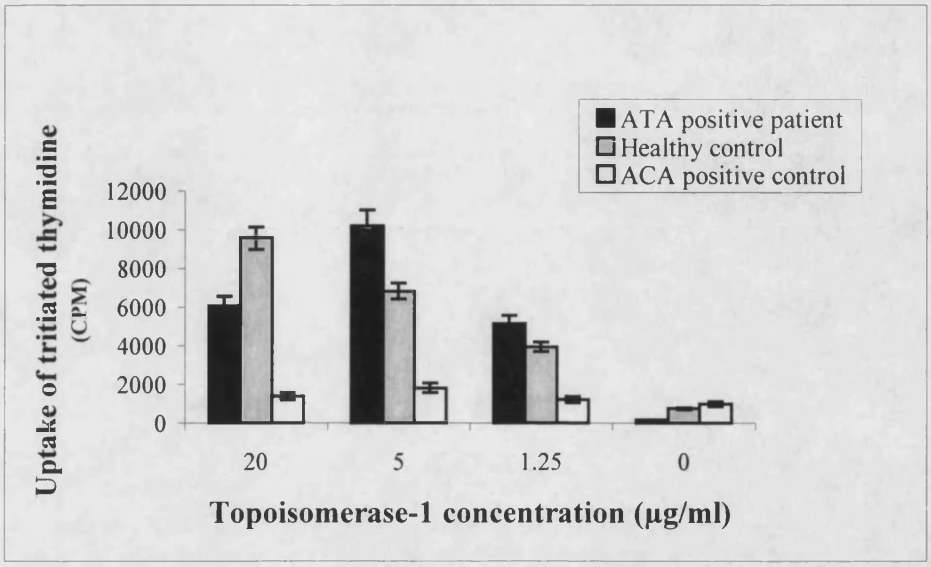
No significant differences were observed when phenotype frequencies of ATA patients also possessing ARA II were compared with those patients who were ATA positive but ARA II negative.

Table 3.7: Comparison of HLA-DRB1 and DQB1 phenotype frequencies within the ARA III group with regard to ARA II

Allele		ARA I/II/III		ARA I/III	
HLA DRB1*		(n=13)	(%)	(n=3)	(%)
0101-0103	(DR1)	5	(38.5)	1	(33.3)
1501-1502	(DR2)	1	(7.7)	1	(33.3)
1601-1602	(DR2)	0	(0.0)	0	(0.0)
Sum DR2		1	(7.7)	0	(0.0)
0301-0303	(DR3)	6	(46.2)	0	(0.0)
0401-0411	(DR4)	4	(30.8)	1	(33.3)
1101-1104	(DR5)	0	(0.0)	0	(0.0)
1201-1202	(DR5)	0	(0.0)	0	(0.0)
Sum DR5		0	(0.0)	0	(0.0)
1301-1305	(DR6)	1	(7.7)	0	(0.0)
1401-1410	(DR6)	1	(7.7)	0	(0.0)
0701-0702	(DR7)	2	(15.4)	2	(66.7)
0801-0805	(DR8)	1	(7.7)	0	(0.0)
0901	(DR9)	0	(0.0)	0	(0.0)
1001	(DR10)	0	(0.0)	0	(0.0)
HLA DQB1*		(n=13)		(n=3)	
0201	(DQ2)	5	(38.5)	2	(66.7)
0401	(DQ4)	0	(0.0)	0	(0.0)
0402	(DQ4)	1	(7.7)	0	(0.0)
0501	(DQ5)	3	(23.1)	1	(33.3)
0502	(DQ5)	0	(0.0)	0	(0.0)
0503	(DQ5)	1	(7.7)	0	(0.0)
0601	(DQ6)	0	(0.0)	0	(0.0)
0602	(DQ6)	1	(7.7)	1	(33.3)
0603/8	(DQ6)	1	(7.7)	0	(0.0)
0604	(DQ6)	0	(0.0)	0	(0.0)
0301	(DQ7)	4	(30.8)	0	(0.0)
0302	(DQ7)	3	(23.1)	1	(33.3)
0303	(DQ7)	0	(0.0)	1	(33.3)

Numbers of individuals for the comparisons made here were too small for analysis to be conclusive (p values for Fishers exact test showed no significant differences). However, an increase in HLA-DRB1*0701-0702 in the ARA I/III group can be observed.

Figure 3.1: Effect of recombinant Topoisomerase I on the proliferation of human lymphocytes



Dose-response curve of mononuclear cells to recombinant topo I at day seven in an anti-topo-I antibody positive SSc patient (■), an anticentomere antibody positive SSc patient (□) and a healthy control (▒). Bars represent range of recordings performed in triplicate. Anti-CD3 responses at day four were greater than eight SI in mononuclear cells from all subjects.

Table 3.8: T-cell proliferation responses to topoisomerase I in patients with connective tissue disease and in healthy controls in relation to MHC Class II status

Study No.	Status	Auto-antibody	T-cell Proliferation (SI)	HLA-DRB1*	HLA-DQB1*	At least 1 allele HLA DQB1: Y: 30 positive
Responder						
1	Normal control	Neg	7.3	0101-0102, 1101-1104	0501, 0301	Yes
2	Normal control	Neg	4	0404, 1101-1104	0302, 0301	Yes (H)
3	Normal control	Neg	3.4	0401, 0404	0302, 0302	Yes (H)
4	Normal control	Neg	10	0701-0702	0602, 0303	Yes (H)
5	Normal control	Neg	9	0707-0711	0201, 0303	Yes
6	SSc	ACA	3.5	1501, 1501	0602, 0602	Yes (H)
7	SSc	ATA	5.7	1501, 0301	0602, 0201	Yes
8	SSc	ATA	3	0901, 1101-1104	0301, 0303	Yes (H)
9	SSc	ATA	5	0101-0102, 1301-1302	0501, 0603/8, (&0201)	No
Non-responder						
10	SSc	Anti-Pm-Scl, anti-Ro	2	1501, 0301	0201, 0201	No
11	SLE	Anti-U1RNP	2	1501, 0404	0602, 0201	Yes
12	SLE	Neg	1.1	0301, 0401-0411	0201, 0302	Yes
13	SLE	Anti-U1RNP	1.6	0401, 1201-1202	0201, 0301	Yes
14	SSc	ACA	1.9	0101, 0101	0501, 0201	No
15	SSc	ACA	2.8	1302, 0401	0604, 0301	Yes
16	SSc	ACA	2.1	0401-0411, 1301-1302	0603/8, 0301	Yes

H = homozygous for HLA-DQB1: Y: 30.

Table 3.9: Distribution of responders and non-responders with regard to HLA-DQB1:30: Y and HLA DR4 status

HLADQB1/DRB1 status	Responders		Non-responders	
	Bath (n = 9)	Japanese (n = 42)	Bath (n = 7)	Japanese (n = 15)
DQB1: 30:Y positive (+/+ or +/-) and DR4 negative (-/-)	**6**	**37**	0	3
DQB1: 30:Y positive (+/+) and DR4 positive (+/+ or +/-)	**2**	**2**	0	2
DQB1: 30:Y positive (+/-) and DR4 positive (+/+ or +/-)	0	2	**5**	**6**
DQB1: 30:Y negative (-/-) and DR4 negative (-/-)	1	1	**2**	**4**

The distribution of individuals according to the proposed model where: The presence of an HLA-DQB1: 30:Y allele is necessary for the production of anti-topo I. The response is suppressed in the presence of HLA-DR4 alleles except where there is homozygosity for DQB1: 30:Y antibodies in which case the suppressive effect of DR4 alleles is overcome. The highlighted quadrants show the individuals falling in the appropriate categories in relationship to their T-cell response/non-response and HLA type. In total 87% (64/73) individuals from the current study and the data reported from a Japanese population (172) fall into the appropriate categories defined by this model. The presence of the expected allele combination favouring a response was present in 47 of 51 responders and in 5 of 22 non-responders ($p < 0.001$).

3.5: DISCUSSION

SSc is characterised by a highly specific autoantibody response that is directed to selective intracellular antigens. Furthermore, there are three main serologically defined subgroups of SSc that are mutually exclusive and distinguish clinical subsets (64). Therefore, it may be expected that the rather weak genetic associations reported for scleroderma in general become stronger if autoantibody-defined clinical subgroups are considered. One of the major autoantibody responses in SSc is to topoisomerase I (topo I). An autoantibody response to topo I defines a group of scleroderma patients with a high prevalence of lung disease (63;64,175;176). Of interest, ATA are also found in high frequency in silica-associated scleroderma where lung disease is extremely common (135). Genetically determined factors including immune response genes may be important in influencing disease susceptibility to an environmental trigger as has been observed with a vinyl chloride induced scleroderma-like syndrome (144). The study of an autoantibody-defined subgroup may define a common genetic background regardless of whether any causative environmental agent has been identified or not.

Previous studies have shown associations of MHC class II alleles and the presence of ATA (63;64;80;84;85;171;177). The most commonly associated allele with ATA is HLA-DR5 originally reported in association with the diffuse form of the disease (63) and found in 70% of ATA positive patients studied by Genth *et al.* (80) and in 82% of anti-topo I patients studied by Morel *et al.* (171).

Significant associations of HLA-DRB1 and DQB1 alleles with ATA have been identified in this study. The results confirm the commonly reported association of HLA-DR5. More recent studies have reported an increase in the HLA-DR11 subtype of HLA-DR5 and in particular an association with the HLA-DRB1*1104 allele (64;171). These results also support an association with HLA-DRB1*1101-1104 alleles rather than with the HLA-DR12 subtype (DRB1*1201-1202). No significant association with HLA-DR2 was observed in the ATA group, as has been previously reported (166;177). Also, the reduction in HLA-DR4 that was noted in the study by Morel et al (171) was not observed in this study. A negative association of HLA-DRB1*0301-0303 was observed in ATA positive patients compared to ATA negative patients, ($p<0.01$), an association which has not been reported before.

There have been more controversial reports of associations between HLA-DQB1 alleles and ATA. The previously reported association of HLA-DQB1*0301 with ATA (31;84;171) was confirmed. There was also a significant association identified with HLA-DQB1*0602 in ATA positive individuals. However, these results could not confirm the previously reported negative association of HLA-DQB1*05 in ATA positive patients compared to ATA negative patients reported by Fanning *et al.* (64).

As mentioned in the introduction to this chapter, there is only one report that includes HLA associations with ARA. There were no striking HLA associations identified in the ARA III group, which is in agreement with the report by Fanning *et al.* (64).

At the amino acid level, Reveille *et al.* reported that all ATA positive patients had an HLA-DQB1 allele encoding for the amino acid tyrosine at position 30 at the second hypervariable domain and an increase in the TRAELDT sequence at positions 71-77 of the third hypervariable domain. The association between HLA-DQB1: 30:Y alleles and ATA has been supported (178), however, more recent studies seem to confirm the association with the HLA-DQB1: ^{71TRAELDT77} sequence but not with the DQB1: 30:Y allele (64;171). The results here indicate significant increases in HLA-DQB1: 30:Y, alleles, and, in agreement with other studies (64;84;171), there was an increase in the HLA-DQB1: ^{71TRAELDT77} sequence. The increase in HLA: DQB1: 26:Y alleles, as reported in a Japanese population Kuwana '93 (85), was also found in this study. None of these loci were significantly associated with the ARA III group.

With regard to the investigation of existing autoantibody group heterogeneity, no HLA associations were identified when ATA and ARA III subgroups were considered. Comparisons made in this study could not confirm findings reported by Fanning *et al.* regarding a significant increase in HLA-DQB1*0201 in ARA I/II/III patients compared to ARA I/III positive patients (64)

A modest difference in HLA background was identified in the mutually exclusive autoantibody groups considered here. HLA-DQB1: 30:Y was significantly increased in ATA positive patients compared to ARA III patients ($p<0.05$). No other significant differences were observed in allele frequencies or in the frequencies of the amino acid loci considered. Thus, in this study, mutual exclusivity of these autoantibody groups

could not be explained by HLA-DRB1 or DQB1 background. That is not to say that the observed mutual exclusivity is not determined by HLA background; associations with alternative loci, such as DPB1, or a combination of loci need to be examined in order to fully assess HLA contribution to autoantibody mutual exclusivity.

Recent studies have been able to demonstrate T cell responses to some of the autoantigens commonly targeted by patients with related autoimmune rheumatic diseases. For instance T cell proliferative responses to small ribonucleoproteins (179) and nucleosomes have been identified (180). Of interest these responses were present not only in patients with the corresponding autoantibody but were present in healthy individuals of a certain MHC class II background (179). Of direct relevance to the current study Kuwana *et al.* reported T cell responses to recombinant forms of topo I in patients with anti-topo I antibodies and in some healthy controls (85). In the latter study DRB1*1501/2, DRB1*1101,03,04 and/or DRB1*07 alleles were common to all responders regardless of disease. Kuwana's group went on to show that HLA-DR-restricted direct contact between CD4 + T cells and B cells was important for anti-topo I antibody synthesis in an *in vitro* culture system (46).

In the present study proliferative mononuclear cell responses to topo I were not confined to those patients who had anti-topo I antibodies present in the serum but were also demonstrable in normal individuals which is similar to the findings of Kuwana *et al.* (172). Unlike Kuwana *et al.* (172) two of the eight responders did not have HLA-DR15, DR11 or DR7 related alleles. However, there was a non-significant increase in HLA-DRB1*1101-4 alleles in responders that together with the results from the autoantibody association studies suggests that at least HLA-DR11 related alleles may facilitate a T cell-driven ATA response. Also noteworthy was a significant increase in homozygosity for HLA-DQB1: 30:Y alleles in responders and a trend for a reduction in HLA-DR4 in non-responders. Intriguingly, a model in which HLA-DQB1: 30:Y alleles facilitate a topo I response and HLA-DR4 suppress such a response could account for 88% of patients reported here and in the report from Kuwana *et al.* (172). This reverse situation in which HLA-DR alleles protect from HLA-DQ mediated effects has some support from studies of collagen-induced arthritis in HLA-DQ8 transgenic mice (181;182).

Our finding of T cells in healthy individuals proliferating to topo I is consistent with other reports of T cell responses to intracellular autoantigens reported by Kuwana *et al.* (172) as mentioned above. The presence of such autoreactive T cells suggests either a breakdown of T cell tolerance to topo I in certain individuals or more likely a failure to establish tolerance during the process of thymic maturation. Topo I is a highly conserved DNA binding protein that is not normally secreted or expressed on the cell surface. However it has been shown that certain intranuclear particles may be translocated to the cell surface during the process of apoptosis (183). In addition, topo I appears to be susceptible to cleavage by reactive oxygen species in a metal cation-dependent manner (43). Such modification, perhaps driven by the generation of reactive oxygen species as a result of ischaemia reperfusion injury, may result in uniquely expressed cryptic epitopes becoming exposed to the immune system initiating a T cell driven immune response. The context in which apoptosis occurs may determine the type of autoantigen fragment generated (154), and set the necessary conditions for T and B cell collaboration leading to autoantibody generation.

The consistent finding of an association between anti-topo I antibody responses and HLA-DR5 associated alleles is supported in the present study. HLA associations have been identified in patients with regard to their autoantibody status and these associations were stronger when particular amino acid loci were considered. However, the relative importance of HLA-DR and HLA-DQ gene complementation remains unclear. The effect of environmental triggers associated with SSc on autoantigen processing and fragmentation is an area worth further study considering the close associations between anti-topo I antibody production and clinical, immunogenetic and environmental subgroups of disease.

CHAPTER 4

ETHNIC VARIATION IN THE SYSTEMIC SCLEROSIS AUTOIMMUNE RESPONSE

4.1: INTRODUCTION

Autoantibody frequency, mutual exclusivity and co-existence in SSc

Autoantibodies are detected in more than 90% of SSc sera (24). The majority of these sera contain autoantibody specificities to one of the three main serologic autoantibody groups: ACA, ATA or ARA III. Typically, ACA is reported to occur in some 28% of SSc patients (45;63;184;185), and ATA and ARA III are detected in approximately 25% (45;63). These three classes of autoantibody demonstrate disease specificity and as discussed in chapter three, are generally considered to be mutually exclusive (64;85).

There are also other, more minor, serologic groups occurring in SSc sera, which do not demonstrate such mutual exclusivity. These include anti-Pm-Scl antibodies, which occur in around four percent of patients, anti-U1RNP, detected in approximately six percent of patients (45;185) and anti-Ro antibodies occurring in up to nine percent of patients (45). Anti-Ro and ATA are often seen to accompany one another in the same serum (45). Anti-U3RNP antibodies have been reported to occur in approximately seven percent of SSc patients (45;186).

Other autoantibody specificities, including ARA II, ARA I and anti-La are not disease specific and frequently (and characteristically) accompany each of the three main SSc-associated autoantibodies. Overall, ARA III, ATA, ACA, anti-Pm-Scl and anti-U3RNP are considered mutually exclusive, with rare exceptions. Anti-Ro and anti-U1RNP are reported to not normally occur with anti-U3RNP, ARA III or ACA (For review see Harvey and McHugh '99 (45))

Autoantibody subgrouping of patients for the current study

For the purpose of the study in this chapter, patients were grouped according to the autoantibody(ies) for which they tested positive. Because many patient sera contained multiple autoantibody specificities, it was necessary to stratify sera according to autoantibody status to enable constructive analysis to be carried out. Specificities comprising one of the major autoantibody groups (ACA, ATA, ARA III) were given first ranking followed by anti-U3RNP, anti-Ro, anti-Pm-Scl, anti-Jo-1, anti-U1RNP, anti-La, and sera lacking detectable autoantibody. Patients were grouped according to first level of stratification.

Racial differences in the frequencies of scleroderma related autoantibodies and disease subtype

As mentioned, several SSc-ANAs are highly disease specific and are associated with clinical subgroups of disease. It has been demonstrated that ethnic variability can influence the type of SSc that a given patient will develop (81), and thus, unsurprisingly, the frequencies of autoantibodies are reported to vary with regard to racial differences (24). The most striking variations are seen in the ACA and anti-U3RNP serological groups. ACAs are associated with the limited form of the disease and are considerably decreased in Blacks compared to Caucasian SSc patients (23;187). Conversely, anti-U3RNP autoantibodies, a minor serologic group associated with severe disease, are detected more frequently in Black than in Caucasian SSc patients (24;186;187).

HLA-SSc associations and race

Ethnic variation in the study population is one factor, which may contribute to the lack of consensus of HLA alleles associated with scleroderma. HLA frequencies associated with SSc do vary in different races. For Caucasoids, there is general consensus for DR1, DR3 and DR5 association with SSc (26). In contrast, for the Japanese, HLA DR2 and DR4 are most frequently reported (169). However, when autoantibody suubgroups are considered HLA associations become more defined. ATA is often reported in association with DR5 (81), and ACA with DR1 (86). Of consideration in the present study is whether the differences in HLA frequencies can explain the variance in ANA incidence and disease subtype, in alternative races.

4.2: CHAPTER AIMS

The objectives of the work described in this chapter were; to identify any similarities and differences of SSc disease profile in two races, and to identify any HLA characteristics that are associated with the above observations, which may further support the role of HLA in SSc. The investigation was carried out by means of the aims set out below:

I. Analysis and comparisons of autoantibody specificities of British Caucasian and South African Black SSc patient populations.

II. Analysis and comparisons of specific clinical manifestations in the two races and subsequent consideration of autoantibody and HLA-DRB1 and/or DQB1 association.

III. To identify any associations of HLA-DRB1 and/or DQB1 alleles with autoantibody subgroup in South African Black SSc patients and a comparison with a British Caucasian SSc population.

4.3: MATERIALS AND METHODS

Patients

Forty-five South African Blacks (SAB) and 229 British Caucasians (BC) fulfilled the established criteria for SSc (156) and were included in the study detailed in this chapter.

Detection of autoantibodies

The presence of serum autoantibody was investigated in all of the patients. Patient sera were screened for anti-cytoplasmic antibody, ANA and ANoA reactivities by IF (method 2.4.2). ANA and ANoA positive sera were further examined by ^{35}S-immunoprecipitation (method 2.4.4) to identify autoantibody specificities. Autoantibody patterns precipitating only a single band, and when autoantibody specificities precipitating multiple bands were observed but without a known positive control sera on the same gel, the results were confirmed by Ouchterlony double immunodiffusion (method 2.4.3) (with the exception of ARA). For each method, comparisons were made with prototype reference sera.

HLA-DRB1 and HLA-DQB1 typing

HLA-DRB1 and HLA-DQB1 alleles were identified by PCR-SSP genetic typing according to method 2.2.2. Details regarding the patients from whom DNA was available will be included in the relevant sections.

Statistical analysis.

Statistical analyses were performed according to method 2.5

4.4: RESULTS

4.4.1: Analysis and comparisons of autoantibody status of British Caucasian and South African Black SSc patient populations

Prevalence of autoantibodies in the South African Black (SAB) and British Caucasian (BC) SSc patient populations

Autoantibody status was investigated in 45 SAB and 229 BC SSc patients. The number of patients testing positive for each autoantibody is represented in the histogram (Figure 4.1).

Eleven autoantibody specificities were identified in the SAB patients, however, 22% of patients precipitated unidentified autoantibody/ies/ only. Anti-Ro was the most frequently detected identified autoantibody (22%). Seven percent of patients lacked detectable serological autoantibody. In the BC SSc patient population the same eleven autoantibody specificities and anti-Jo-1 were identified. Only eight percent of patients possessed an autoantibody that was not identified. The most frequently detected autoantibody in this population was ACA. Eight percent of patients lacked any detectable autoantibody.

When the two populations were compared significant differences in the frequency of autoantibodies were apparent. The greatest difference was identified in the frequency of anti-U3RNP. The presence of this autoantibody was significantly raised in SAB compared to BC SSc patients (<1% BC vs. 17.8% SAB, $p<0.0083$). Figure 4.2 is an example of the immunofluorescence pattern of this autoantibody seen in the SAB patients. ACA autoantibody frequency also showed significant variation between the two populations. This autoantibody was detected in 39% of BC patients compared to nine percent of SAB patients ($p^{corrected}=0.0002$). Figure 4.3 shows the classic immunofluorescence pattern of ACA positive sera, and is an example from a BC patient. Other significant differences were identified in the anti-U1RNP group, which was increased in SAB compared to BC (18% *vs.* 7% respectively, $p^{corrected} <0.05$), ARA II showed an increase in BC compared SAB (15% *vs.* 2.5% respectively, $p^{corrected} <0.05$), and anti-Ro, which was increased in SAB compared to BC (22% *vs.* 7% respectively, $p<0.05$).

Patient autoantibody specificity and mutual exclusivity in BC and SAB populations

Patients were classed into serological groups according to their autoantibody profile, as explained in the introduction to this chapter, and details are shown in table 4.1. Figures 4.4 and 4.5 show the numbers of patients in each serological group for BC and SAB patient populations, respectively. Sixty seven percent of Caucasian patients were included in the three 'major serological groups' compared with only 26.5% of SAB patients ($p<0.0001$). Details of the remaining serological groups are contained in table 4.1. For Caucasians, the largest serological group was ACA (39%). (This was expected as this autoantibody was most prevalent in this population and comprises one of the major serological groups). Thirty-seven patients (17%) were ATA positive, and five of these patients were also anti-Ro positive. Ten patients were ATA and ARA II positive (which included one of the patients who was anti-Ro positive). Twenty seven (12%) of patients were ARA III positive. One of the patients in this group was also anti-Ro positive. In the SAB population the largest serological group was the anti-U3RNP group comprising 8 (18%) patients. The second largest groups were anti-Ro and ATA, each containing 6 (13%) patients.

A variety of combinations of autoantibody specificities were identified in each population. However, it can be seen that, (with the exception of one patient), the observed mutual exclusivity of autoantibodies is in concordance with reports, in both populations. ACA, ATA and ARA III do not occur together in either the BC or SAB patients. ARA III, ATA, ACA, anti-Pm-Scl and anti-U3RNP are also all mutually exclusive in the individuals studied here. ARA III and ACA are not seen to occur with anti-Ro or anti–U3RNP, however, unusually anti-Ro and anti-U3RNP were both identified in the serum from one patient.

Unidentified autoantibodies

Both populations included patients whose sera contained autoantibodies, which were not identified, and a significantly greater number were detected in the SAB patient group (BC 8%, SAB 22%, $p=0.008$). Unidentified immunoprecipitation patterns varied in the BC population, but in the SAB patients some showed similar patterns to each other, suggesting the same autoantigen(s).

4.4.2: Analysis and comparisons of specific clinical manifestations in a British Caucasian and a Black South African population, and subsequent consideration of autoantibody and HLA association

Although it would be appropriate to consider a range of clinical manifestations in this study, sufficient clinical data were only available from both populations for disease subtype and pulmonary fibrosis. These two manifestations will be considered presently.

Disease Subtype in the two races

In the BC cohort 78% (170/217) patients were defined as having lcSSc, thus the remainder, 22%, had dcSSc. In the SAB population 36% (15/42) patients were defined with lcSSc and the remaining 64% dcSSc. These results show a significant increase in dcSSc in SAB compared to BC ($p<0.0001$).

Serological autoantibody association with disease subtype

In BC SSc patients ACA was significantly associated with the lcSSc ($p<0.0001$). Ninety eight percent (83/85) of individuals who were ACA positive had limited disease. In contrast, 77% (20/26) of BC patients in the ARA III group had dcSSc, and there was also a significant association of this autoantibody with diffuse disease ($p<0.0001$, $p<0.0005$ B.$^{Corr.}$). The distribution of patients in the ATA group was fairly even with respect to disease subtype, 49% (16/33) dcSSc, 51% lcSSc, however, overall dcSSc was associated with ATA positivity ($p<0.0001$). All patients in the anti-PmScl group ($n=8$) and the anti-U1RNP group ($n=12$), and 89% (8/9) patients in the anti-Ro group had lcSSc, although these autoantibody groups were not significantly associated with either disease subtype. The majority of patients with unidentified autoantibody(ies) and autoantibody negative patients had lcSSc (77% and 71% respectively).

In SAB, disease subtype was not significantly associated with any of the autoantibody groups.

Serological autoantibody association with pulmonary fibrosis

Clinical information regarding pulmonary fibrosis (PF) was available from 38/45 of the SAB patients, 26% (10/38) of whom had PF. Twenty eight percent did not show any clinical signs of interstitial lung disease. Autoantibody specificities of the patients from

whom details regarding PF were not available included, 1 patient with unidentified autoantibody specificity, 4 patients in the anti-U1RNP serological group, and 2 patients in the anti-U3RNP serological group. Fifty percent of SAB patients with PF were classed in the unidentified-autoantibody serological group and PF was significantly associated with this serological group (p=0.03 Fishers exact test). However significance was lost when Bonferroni correction was applied (p=0.12). Of interest, 3/4 patients positive for ATA only, (i.e. did not possess any other, accompanying, autoantibody(ies)), had PF. The remaining two patients with PF included one anti-U1RNP and one anti-U3RNP positive SSc patient.

Clinical information regarding pulmonary fibrosis (PF) was available on 97/100 (97%) BC patients. (The three BC patients for whom PF was not defined were ATA positive). Thirty five point five percent (34) BC patients had PF. The ACA autoantibody group showed a negative association with PF (p<0.0001), and in contrast, ATA autoantibody was significantly associated with PF (p=0.02). No further significant associations were identified in any of the other autoantibody groups in the BC SSc population.

4.4.3: HLA associations with SSc, and within SSc autoantibody subgroups, in South African Black and British Caucasian SSc patients

Comparison of HLA-DRB1 and DQB1 phenotype frequencies of British Caucasian and South African Black SSc patients with their respective, ethnically matched, normal control population.

Allele frequencies of SSc patients varied significantly, in both ethnic groups, when compared to their respective ethnically matched control population (table 4.4). BC SSc patients showed a significant increase in HLA-DRB1*0301-0303 (BC SSc 64/179 *vs* 53/250 BC controls, p=0.01 with B. corr.) and significant decreases in alleles comprising HLA DQ6 and DQ3 (BC SSc 59/179 *vs* 115/250 BC controls and BC SSc 100/179 *vs* 173/250 BC controls, p= 0.05 with B. corr.), respectively. In contrast to these findings, SAB SSc patients showed a significant increase in HLA DR2 and more specifically in the HLA-DRB1*1501-1502 alleles compared to SAB controls (SAB SSc patients 11/40 *vs* 6/86 SAB controls, p=0.05 with B. corr.). HLA-DRB1*1301-1305

was also significantly increased in the SAB SSc patients compared to the SAB control population (SAB SSc patients 17/40 *vs* 15/86 SAB controls, p=0.03 with B. corr.).

HLA-DQB1 typing for the SAB SSc patients was of low resolution, thus for analysis to be carried out effectively all other populations have been divided according to the same level of specificity. Significant differences in allele frequencies were identified in the SSc populations from both ethnic groups when they were compared to their respective control populations. HLA DQB1*0301 and DQ6 were significantly decreased in the BC SSc patients compared to BC Controls (BC SSc 59/179 *vs* 115/250 BC controls, p=0.01 with B. corr.). In the SAB SSc population HLA DQB1*0501-0504 was significantly increased compared to the SSc control population (SAB SSc patients 13/40 *vs* 11/86 SAB controls, p=0.03 with B. corr.).

Comparison of HLA allele frequency with regard to autoantibody subgroup

For each autoantibody subgroup, comparisons of HLA frequencies were made, within each population, (autoantibody positive compared with the respective remaining (autoantibody) negative group, for that population). A comparison of the findings for each ethnic population will be made and discussed at the end of this chapter. The HLA frequencies of ATA and ARA III autoantibody subgroups in the BC population have been investigated in chapter 3, so the details will not be repeated here.

HLA and ATA

Table 4.5 shows comparisons of HLA-DRB1 and DQB1 allele phenotype frequencies of ATA positive with ATA negative SAB SSc patients. No significant differences in phenotype frequencies were identified between the SAB ATA positive group and the SAB ATA negative group, however this group was small, (n=6). The equivalent data for the BC group is shown in table 3.1 in chapter 3, where the details are discussed.

HLA and ACA

HLA details for ACA positive and ACA negative, SAB and BC SSc patients are shown in table 4.6. The SAB ACA positive group was too small for any meaningful statistical analysis. However, Fishers exact test indicated that there were no significant differences in HLA allele frequency between the SAB ACA positive and negative groups. Significant differences were detected in the BC SSc patients group. HLA-DRB1*0101-

0104 was significantly increased in ACA positive compared to ACA negative patients (BC ACA+ 42% vs. 23% BC ACA-, p=0.009). HLA-DRB1*0401-0411 was also increased in BC ACA positive patients compared to the respective negative group (BC ACA+ 47% vs. 29% BC ACA -, p=0.01; p=0.13 with B.^{corr}). HLA-DRB1*0301-0303 was significantly decreased in BC ACA positive compared with ACA negative patients (BC ACA+ 20% vs. 45% BC ACA-, p=0.001, p=0.013 with B.^{corr}).

With regard to the DQB1 locus, HLA-DQB1*0501-0504 and *0401-0402 were increased in BC ACA positive compared to BC ACA negative patients (ACA+ 45% vs. 28% ACA-, p=0.01; ACA+ 10% vs. 2% ACA-, p=0.03) respectively, however, significance was lost for both loci when Bonforroni correction was applied.

HLA and anti-U3RNP

The number of patients comprising the anti-U3RNP serological group was small, thus statistical analysis was not considered conclusive (table 4.7). However, HLA DR5 was increased in SAB anti-U3RNP positive patients compared to SAB anti-U3RNP negative patients (p=0.04). Of note, both of the BC anti-U3-RNP positive patients were HLA-DRB1*0301 and HLA-DQB1*0201 positive.

HLA and anti-Ro

When HLA-DRB1 allele phenotype frequencies of SAB patients in the anti-Ro serological group were compared with patients not in this group, some differences were observed, however they were not significant. HLA-DRB1*0301-0303 and 1001 were increased in SAB anti-Ro positive patients (p=0.07 and 0.09 respectively), and HLA DR5 was decreased in SAB anti-Ro positive patients (p=0.07). One significant difference identified in the BC anti-Ro serological group was a significant increase in HLA-DRB1*0301-0303 (p=0.04). No significant differences in HLA-DQB1 frequency were observed in the SAB or BC patients in the anti-Ro serological group compared to patients not in this group. The results are presented in table 4.8.

HLA and ARA III serological group

Only two SAB patients belonged to the ARA III serological group and they had different HLA types. The BC ARA III HLA details are considered and discussed in chapter 3.

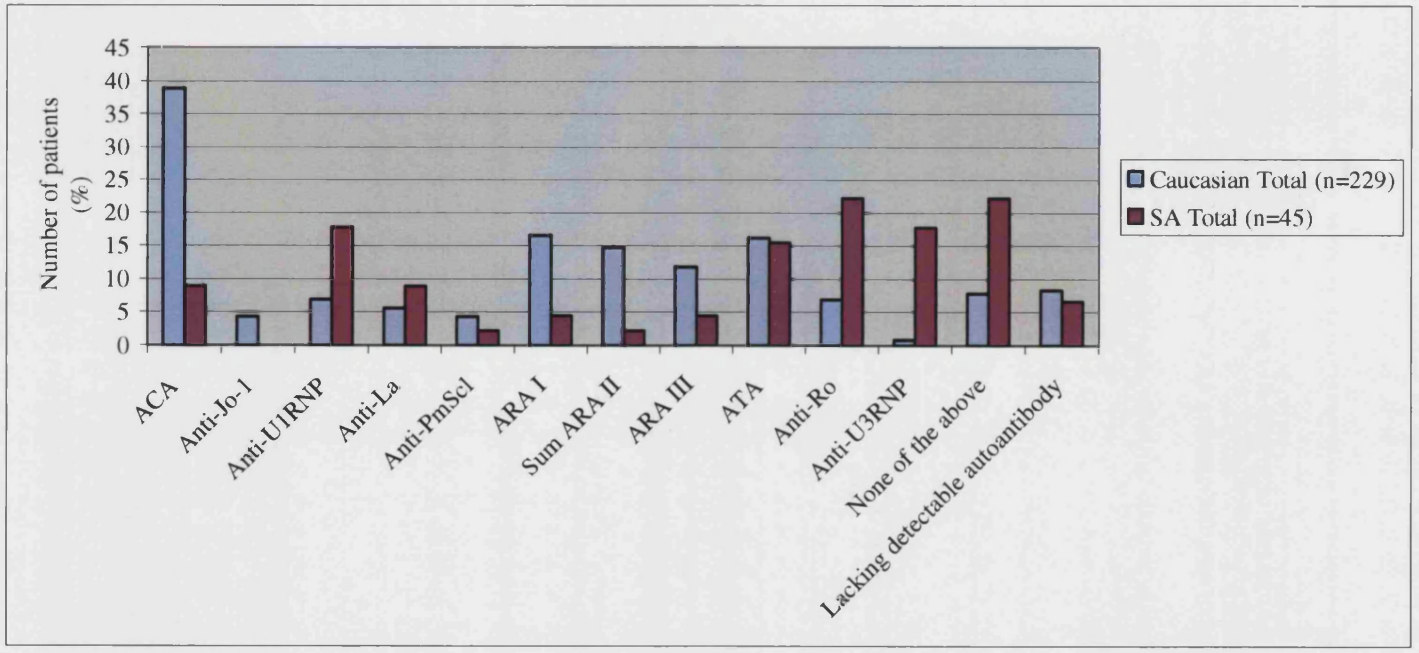
HLA and disease subtype

No significant HLA associations with disease subtype were observed. In the SAB patient population HLA-DRB1*1001 and HLA-DQB1*0201-0203 were raised in lcSSc patients compared to dcSSc (lcSSc 4/15 *vs.* 0/23, p=0.01 and 5/15 *vs.*1/23, p=0.02 respectively), however, when Bonforroni correction was applied these values were no longer significant. HLA-DRB1*0401-0411 was increased to some degree in lcSSc compared to dcSSc BC patients (p=0.06).

HLA and pulmonary fibrosis

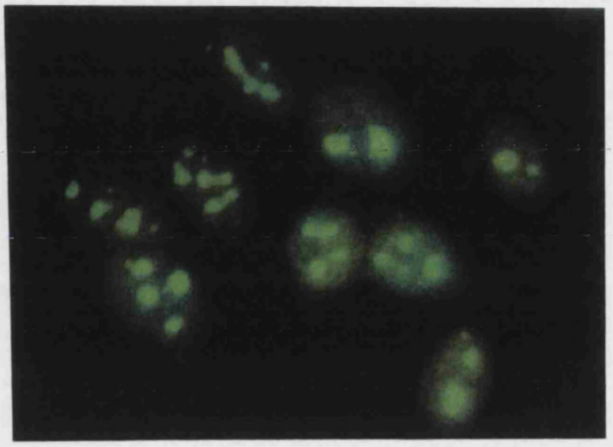
No significant associations with HLA-DRB1 or DQB1 alleles were identified in SAB or BC SSc PF positive patients compared with PF negative patients in each respective race. However, HLA DR5 was increased in SAB PF patients compared to SAB patients without PF.

Figure 4.1: Prevalence of autoantibody specificities in South African Black (SAB) and British Caucasian (BC) SSc patients



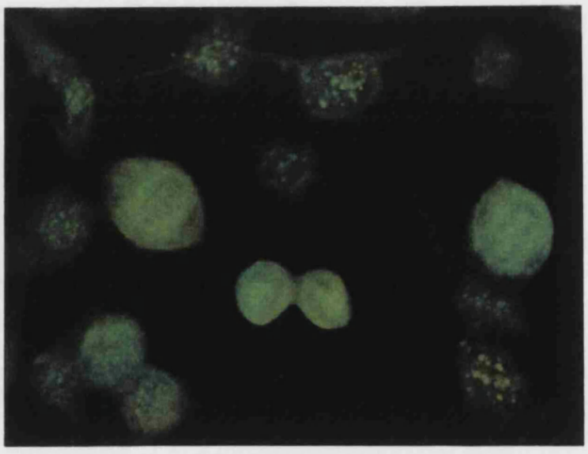
Percentages of South African Black and British Caucasian SSc patients recognising identified and unidentified autoantibody specificties, and those in which no autoantibodies were detected. The greatest differences in autoantibody prevalence between populations were observed in the anti-U3RNP and ACA groups ($p<0.0083$ and $p^{corrected}=0.0002$ respectively). Other significant differences were in anti-U1RNP and ARA III (both $p^{corrected}<0.05$), and anti-Ro ($p<0.05$).

Figure 4.2: Indirect immunofluorescence pattern produced by an anti-U3RNP positive, South African Black, SSc patient



A clumpy, nucleolar, fluorescence pattern, characteristic of anti-U3RNP positive sera

Figure 4.3: Indirect immunofluorescence pattern produced by an ACA positive, British Caucasian, SSc patient



ACA fluorescence; a pattern of discrete dots in interphase cells that line up on metaphase plate in dividing cells

Table 4.1: Autoantibody specificities of South African Black and British Caucasian SSc patients, and classification according to serological group

Autoantibody Specificy(ies)	British Caucasian				South African Black				Serological group
	No. of patients (n=229)	(%)	Total in serological group (n=229)	(%)	No. of patients (n=45)	(%)	Total in serological group (n=45)	(%)	
ACA	88	36.8			3	6.7			
ACA, Positive undentified	1	0.4	89	38.9	1	2.2	4	8.9	**ACA**
ATA	22	9.2			4	8.9			
ARA II, ATA, Anti-Ro	1	0.4			0	0.0			
ATA, ARA II, Anti-Jo-1	1	0.4			0	0.0			
ATA, ARA II	8	3.4			1	2.2			
ATA, Anti-Ro	2	0.85	37	16.2	0	0.0	6	13.3	**ATA**
ATA, Anti-Ro, Anti-Jo-1	1	0.4			0	0.0			
ATA, Anti-Ro, Anti-La	1	0.4			0	0.0			
ATA, Anti-Ro, Anti-La, Anti-U1RNP	0	0.0			1	2.2			
ATA, Anti-U1RNP +/-sm	1	0.4			0	0.0			
ARA I/III	6	2.5			1	2.2			
ARA I/II/III	20	8.4			0	0.0			
ARA III, Ro	0	0.0	27	11.8	1	2.2	2	4.5	**ARA III**
ARA III	1	0.4			0	0.0			
Anti-U3RNP, Positive undientified	0	0.0			2	4.5			
Anti-Ro, Anti-La, Anti-U3RNP	2	0.85	2	0.9	0	0.0	8	17.8	**Anti-U3RNP**
Anti-U3RNP	0	0.0			6	13.3			

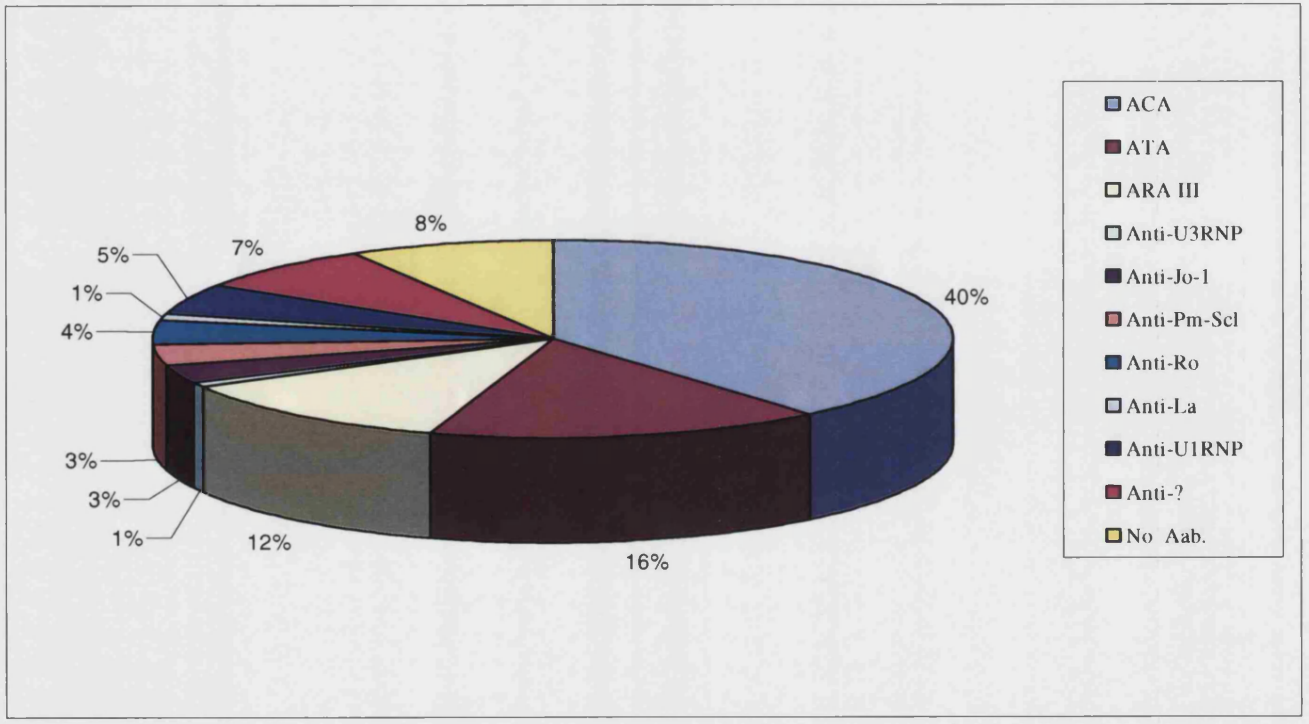
Continued

Table 4.1 continued:

Autoantibody Specificy(ies)	British Caucasian				South African Black				Serological group
	No. of patients (n=229)	(%)	Total in serological group (n=229)	(%)	No. of patients (n=45)	(%)	Total in serological group (n=45)	(%)	
Anti-Jo-1	6	2.5			0	0.0			
Anti-Jo-1, Anti-U1RNP +/-sm	1	0.4	7	3.1	0	0.0	0	0.0	**Anti-Jo-1**
Anti-La, Anti-Pm-Scl	1	0.4			0	0.0			
Anti-Pm-Scl, positive unidentified	0	0.0	8	3.5	1	2.22	1	2.23	**Anti-Pm-Scl**
Anti-Pm-Scl	7	2.9			0	0.0			
Anti-Ro	1	0.4			2	4.5			
Anti-Ro, Anti-La	4	1.7			2	4.5			
Anti-Ro, Anti-La, Anti-Jo-1, Anti-U1RNP +/-sm	1	0.4			0	0.0			
Anti-Ro, Anti-La, Anti-Pm-Scl	1	0.4	9	4.0	0	0.0	6	13.33	**Anti-Ro**
Anti-Ro, Anti-Pm-Scl	1	0.4			0	0.0			
Anti-Ro, Anti-La, Anti-U1RNP	0	0.0			1	2.2			
Anti-Ro, Anti-U1RNP	1	0.4			1	2.2			
Anti-La	2	0.85	2	0.9	0	0.0	0	0.0	**Anti-La**
Anti-U1RNP +/-sm	12	5.0			4	8.9			
Anti-U1RNP, Positive unidentified	0	0.0	12	5.25	1	2.2	5	11.11	**Anti-U1RNP**
None of the above.	17	7.1	17	7.4	10	22.3	10	22.22	**Anti-?**
Lacking detectable autoantibody	19	8.0	19	8.3	3	6.7	3	6.67	**No Aab.**

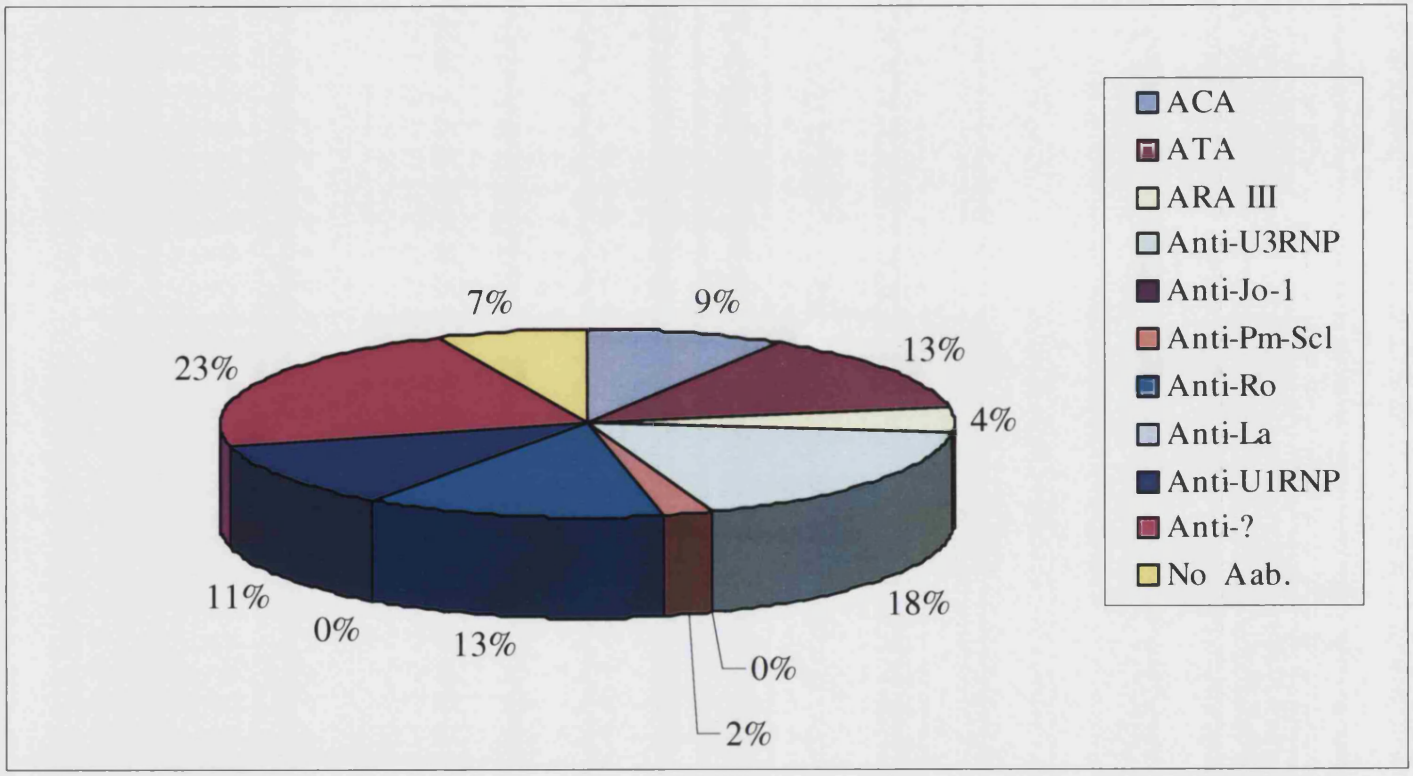
Autoantibody specificities detected in British Caucasian and South African Black SSc patients' serum. Patients have been grouped serologically according to the details described in the text.

Figure 4.4: Serological grouping of British Caucasian SSc patients



The pie chart shows the number of British Caucasian patients in each serological group. The majority of patients (64%) are included in one of the major one of the major serological groups (ACA, ATA, or ARA III).

Figure 4.5: Serological grouping of South African Black SSc patients.



Percentages of Black South African SSc patients' classified into each serological autoantibody group. The largest serological groups were Anti-U3-RNP (18%), and anti-Ro and ATA (both 13%). Only 26% patients were classed into, either the ACA, ATA or ARA III serological groups.

Table 4.2: Serological autoantibody association with disease subtype in British Caucasian SSc and South African Blacks

Aab. Group	BC lcSSc patients (n=167)	(%)	BC dcSSc patients (n=47)	(%)	SAB lcSSc patients (n=15)	(%)	SAB dcSSc patients (n=27)	(%)
ACA	83[1]	(49.7)	2	(4.3)	1	(6.7)	2	(7.4)
ATA	17[3]	(10.2)	16	(34.0)	1	(6.7)	5	(18.5)
ARA III	6[2]	(3.6)	20	(42.6)	0	(0.0)	2	(7.4)
Anti-U3RNP	2	(1.2)	0	(0.0)	3	(20.0)	4	(14.8)
Anti-Jo-1	6	(3.6)	1	(2.1)	0	(0.0)	0	(0.0)
Anti-Pm-Scl	8	(4.8)	0	(0.0)	0	(0.0)	1	(3.7)
Anti-Ro	8	(4.8)	1	(2.1)	3	(20.0)	2	(7.4)
Anti-La	0	(0.0)	2	(4.3)	0	(0.0)	0	(0.0)
Anti-U1RNP	12	(7.2)	0	(0.0)	1	(6.7)	4	(14.8)
Anti-unidentified	13	(7.8)	2	(4.3)	5	(33.3)	5	(18.5)
No Aab.	11	(6.6)	3	(6.4)	1	(6.7)	2	(7.4)

Three serological autoantibody groups showed significant associations with disease subtype in the British Caucasian population, p values with OR(CI) are as follows; [1] $p<0.0001$: 22.2(5.2-94.6), [2] $p^{corrected}<0.0005$: 0.2(0.1-0.5), [3]$p<0.0001$: 0.05(0.01-0.1). No significant associations were identified in the South African Black SSc population. Aab group, serological autoantibody group; BC, British Caucasian; SAB, South African Black; lcSSc, limited cutaneous SSc; dcSSc, diffuse cutaneous SSc.

Table 4.3: Serological autoantibody association with pulmonary fibrosis in British Caucasians and South African Blacks.

Serological Group	BC PF Positive (n=33)	(%)	BC PF Negative (n=64)	(%)	SAB PF Positive (n=10)	(%)	SAB PF Negative (n=28)	(%)
ACA	3[2]	(9.09)	39	(60.94)	0	(0.0)	4	(14.29)
ATA	12[3]	(36.36)	10	(15.63)	3	(30.0)	3	(10.71)
ARA III	7	(21.21)	6	(9.38)	0	(0.0)	2	(7.14)
Anti-U3RNP	1	(3.03)	0	(0.0)	1	(10.0)	3	(10.71)
Anti-Jo-1	1	(3.03)	3	(4.69)	0	(0.0)	0	(0.0)
Anti-Pm-Scl	2	(6.06)	0	(0.0)	0	(0.0)	1	(3.57)
Anti-Ro	3	(9.09)	2	(3.13)	0	(0.0)	6	(21.43)
Anti-La	0	(0.0)	0	(0.0)	0	(0.0)	0	(0.0)
Anti-U1RNP	1	(3.03)	2	(3.13)	1	(10.0)	2	(7.14)
Anti-unidentified	2	(6.06)	1	(1.56)	5[1]	(50.0)	4	(14.29)
No Aab.	1	(3.03)	1	(1.56)	0	(0.0)	3	(10.71)

South African Black patients with unidentified autoantibody(ies) showed a significant association with pulmonary fibrosis; [1] p=0.03: 6(1.2-30). ACA positive British Caucasian patients showed a negative association with pulmonary fibrosis; [2] p<0.0001: 0.06(0.01-0.23), and ATA positive patients had a positive association; [3] p=0.02: 3.08(1.1-8.2). SAB, South African Blacks: BC, British Caucasian: Aab, autoantibody.

Table 4.4: A comparison of HLA-DRB1 and DQB1 phenotype frequencies of British Caucasian SSc patients and South African Black SSc patients with their respective, ethnically matched, control populations.

Allele		BC Controls		BC SSc Patients		SAB SSc Patients		SAB Controls	
HLA DRB1*		(n=250)	(%)	(n=179)	(%)	(n=40)	(%)	(n=86)	(%)
0101-0103	(DR1)	53	(21.2)	55	(30.7)	4	(10.0)	2	(1.7)
1501-1502	(DR2)	65	(26.0)	38	(21.2)	11[4]	(27.5)	6[4]	(6.4)
1601-1602	(DR2)	6	(2.4)	2	(1.1)	0	(0.0)	0	(0.0)
Sum DR2		71	(28.4)	38	(22.2)	11[5]	(27.5)	6[5]	(6.4)
0301-0303	(DR3)	53[1]	(21.2)	64[1]	(35.7)	13	(32.5)	28	(24.3)
0401-0411	(DR4)	88	(35.2)	64	(35.7)	3	(7.5)	5	(4.1)
1101-1104	(DR5)	41	(16.4)	24	(13.4)	10	(25.0)	28	(24.0)
1201-1202	(DR5)	7	(2.8)	3	(1.7)	5	(12.5)	6	(4.7)
Sum DR5		46	(19.2)	27	(15.1)	15	(37.5)	33	(28.7)
1301-1305	(DR6)	54	(21.5)	25	(14.0)	17[6]	(42.5)	15[6]	(13.2)
1401-1410	(DR6)	5	(2.0)	4	(2.2)	1	(2.5)	3	(2.4)
0701-0702	(DR7)	63	(25.2)	34	(19.0)	4	(10.0)	9	(7.6)
0801-0805	(DR8)	13	(5.2)	11	(6.2)	2	(5.0)	4	(3.0)
0901	(DR9)	4	(1.6)	3	(1.7)	0	(0.0)	2	(1.7)
1001	(DR10)	2	(0.8)	2	(1.1)	4	(10.0)	3	(2.4)
HLA DQB1*		(n=250)	(%)	(n=183)	(%)	(n=40)	(%)	(n=87)	(%)
0201-0203	(DQ2)	88	(35.2)	81	(44.3)	7	(17.5)	19	(16.9)
0401-0402	(DQ4)	10	(4.0)	9	(4.9)	10	(25.0)	23	(20.2)
0501-0504	(DQ5)	66	(26.4)	63	(34.4)	13[7]	(32.5)	11[7]	(9.8)
0601	(DQ6)	3	(1.2)	1	(0.6)	0	(0.0)	0	(0.0)
0602-4/8-12/14	(DQ6)	115[2]	(46.0)	59[2]	(32.2)	17	(42.5)	37	(31.8)
0301-4/11-12	(DQ7)	173[3]	(69.2)	100[3]	(54.6)	4	(10.0)	23	(19.6)

Significant differences in allele phenotype frequencies were observed between SSc patients when compared to their respective ethnically matched control population. P values with Odds Ratio and confidence intervals are shown. British Caucasians: [1,] $p^{corrected}$ = 0.01: 0.5(0.3-0.7), [2, 3] $p^{corrected}$ = 0.01: 1.7(1.2-2.7). South African Blacks: [4, 5] $p^{corrected}$ = 0.05: 5.0(1.7-14.9), [6, 7] $p^{corrected}$ = 0.03: 3.4(1.4-8.0). (South African Black control data obtained from 'HLA 1991; Proceedings of the 11th International histocompatibility workshop and conference' (230)).

Table 4.5: HLA-DRB1 and DQB1 phenotype frequencies of ATA positive and ATA negative Black South African SSc patients.

Allele	SAB ATA negative		SAB ATA positive	
HLA DRB1*	(n=34)	(%)	(n=6)	(%)
0101-0103	4	(11.76)	0	(0.0)
1501-1502	8	(23.53)	3	(50.0)
1601-1602	0	(0.0)	0	(0.0)
Sum DR2	8	(23.53)	0	(0.0)
0301-0303	12	(35.29)	1	(16.67)
0401-0411	3	(8.82)	0	(0.0)
1101-1104	9	(26.47)	1	(16.67)
1201-1202	4	(11.76)	1	(16.67)
Sum DR5	13	(38.24)	0	(0.0)
1301-1305	14	(41.18)	3	(50.0)
1401-1410	1	(2.94)	0	(0.0)
0701-0702	3	(8.82)	1	(16.67)
0801-0805	2	(5.88)	0	(0.0)
0901	0	(0.0)	0	(0.0)
1001	3	(8.82)	1	(16.67)
HLA DQB1*	(n=34)		(n=6)	
0201-0203	7	(20.59)	0	(0.0)
0401-0402	8	(23.53)	2	(33.33)
0501-0504	11	(32.35)	2	(33.33)
0601	0	(0.0)	0	(0.0)
0602/9-12/14	21	(61.76)	5	(83.33)
0301-4/11-12	9	(26.47)	1	(16.67)

No significant differences in allele phenotype frequencies were observed between ATA positive and ATA negative Black South African SSc patients.

Table 4.6: A comparison of HLA-DRB1 and DQB1 phenotype frequencies of ACA positive and ACA negative SSc patients; in a Black South African SSc patient population and a British Caucasian SSc patient population

Allele	SAB ACA negative		SAB ACA positive		BC ACA positive		BC ACA negative	
HLA DRB1*	(n=4)	(%)	(n=36)	(%)	(n=69)	(%)	(n=110)	(%)
0101-0103	1	(25.0)	3	(8.33)	29[1]	(42.03)	26[1]	(23.64)
1501-1502	1	(25.0)	10	(27.78)	13	(18.84)	25	(22.73)
1601-1602	0	(0.0)	0	(0.0)	0	(0.0)	2	(1.82)
Sum DR2	1	(25.0)	10	(27.78)	13	(18.84)	25	(22.73)
0301-0303	0	(0.0)	13	(36.11)	14[2]	(20.29)	50[2]	(45.45)
0401-0411	0	(0.0)	3	(8.33)	32	(46.38)	32	(29.09)
1101-1104	0	(0.0)	10	(27.78)	7	(10.14)	17	(15.45)
1201-1202	0	(0.0)	5	(13.89)	1	(1.45)	2	(1.81)
Sum DR5	0	(0.0)	14	(38.89)	8	(11.59)	19	(17.27)
1301-1305	3	(75.0)	14	(38.89)	11	(15.94)	14	(12.73)
1401-1410	0	(0.0)	1	(2.78)	1	(1.45)	3	(2.73)
0701-0702	1	(25.0)	3	(8.33)	0	(14.49)	15	(21.82)
0801-0805	0	(0.0)	2	(1.20)	7	(10.14)	2	(3.64)
0901	0	(0.0)	0	(0.0)	0	(0.0)	1	(2.73)
1001	0	(0.0)	4	(11.11)	1	(1.45)	1	(0.91)
HLA DQB1*	(n=4)		(n=36)		(n=71)		(n=112)	
0201-0203	1	(25.0)	6	(16.67)	33	(46.48)	49	(43.75)
0401-0402	0	(0.0)	10	(27.78)	7	(9.86)	2	(1.79)
0501-0504	1	(25.0)	12	(33.33)	32	(45.07)	31	(27.68)
0601	0	(0.0)	0	(0.0)	1	(1.41)	0	(0.0)
0602/9-12/14	3	(75.0)	14	(38.89)	22	(30.99)	37	(33.04)
0301-4/11-12	0	(0.0)	4	(11.11)	41	(57.75)	59	(52.68)

Significant differences in allele phenotype frequencies were only observed between ACA positive and ACA negative British Caucasians (BC) SSc patients, and not in the South African Black (SAB)ACA positive *vs.* ACA negative population: [1] $p = 0.009$, [2] $p = 0.013$.

Table 4.7: A comparison of HLA-DRB1 and DQB1 phenotype frequencies of anti-U3RNP positive and anti-U3RNP negative SSc patients; in a Black South African SSc patient population and a British Caucasian SSc patient population

Allele	SAB anti-U3RNP negative		SAB anti-U3RNP positive		BC anti-U3RNP positive		BC anti-U3RNP negative	
HLA DRB1*	(n=35)	(%)	(n=5)	(%)	(n=2)	(%)	(n=177)	(%)
0101-0103	4	(11.4)	0	(0.0)	0	(0.0)	55	(31.0)
1501-1502	11	(31.4)	0	(0.0)	0	(0.0)	38	(21.5)
1601-1602	0	(0.0)	0	(0.0)	0	(0.0)	2	(1.1)
Sum DR2	11	(31.4)	0	(0.0)	0	(0.0)	38	(21.5)
0301-0303	13	(37.1)	0	(0.0)	2	(50.0)	62	(35.0)
0401-0411	2	(5.7)	1	(20.0)	0	(0.0)	64	(36.2)
1101-1104	7	(20.0)	3	(60.0)	0	(0.0)	24	(13.6)
1201-1202	3	(8.6)	2	(40.0)	0	(0.0)	3	(1.7)
Sum DR5	10[1]	(28.6)	4[1]	(80.0)	0	(0.0)	27	(15.3)
1301-1305	14	(40.0)	3	(60.0)	1	(50.0)	24	(13.6)
1401-1410	1	(2.9)	0	(0.0)	0	(0.0)	4	(2.3)
0701-0702	4	(11.4)	0	(0.0)	0	(0.0)	34	(19.2)
0801-0805	2	(5.7)	0	(0.0)	1	(50.0)	10	(5.6)
0901	0	(0.0)	0	(0.0)	0	(0.0)	3	(1.7)
1001	4	(11.4)	0	(0.0)	0	(0.0)	2	(1.1)
HLA DQB1*	(n=35)		(n=5)		(n=2)		(n=181)	
0201-0203	7	(20.0)	0	(0.0)	2	(100.0)	79	(43.7)
0401-0402	9	(25.7)	1	(20.0)	1	(50.0)	8	(4.5)
0501-0504	11	(31.4)	2	(40.0)	0	(0.0)	63	(34.8)
0601	0	(0.0)	0	(0.0)	0	(0.0)	1	(0.6)
0602/9-12/14	13	(37.1)	4	(80.0)	1	(50.0)	58	(32.0)
0301-4/11-12	2	(5.7)	2	(40.0)	0	(0.0)	100	(55.2)

A significant association of HLA DR5 with anti-U3RNP was observed in SAB patients; [1] p=0.04, however, the number of individuals positive for anti-U3RNP were few for both populations, thus statistical analysis was considered inconclusive.

Table 4.8: A comparison of HLA-DRB1 and DQB1 phenotype frequencies of anti-Ro positive and anti-Ro negative SSc patients; in a Black South African SSc patient population and a British Caucasian SSc patient population.

Allele	SAB Anti-Ro negative		SAB anti-Ro positive		BC anti-Ro positive		BC anti-Ro negative	
HLA DRB1*	(n=34)	(%)	(n=6)	(%)	(n=8)	(%)	(n=171)	(%)
0101-0103	4	(11.76)	0	(0.0)	2	(25.0)	53	(31.0)
1501-1502	9	(26.47)	2	(26.47)	1	(12.5)	37	(21.64)
1601-1602	0	(0.0)	0	(0.0)	1	(12.5)	1	(0.58)
Sum DR2	11	(32.35)	0	(0.0)	1	(12.5)	37	(21.56)
0301-0303	9	(26.47)	4	(66.67)	6[1]	(75.0)	58[1]	(38.80)
0401-0411	2	(5.88)	1	(16.67)	1	(12.5)	63	(36.72)
1101-1104	10	(29.41)	0	(0.0)	2	(25.0)	22	(12.82)
1201-1202	5	(14.71)	0	(0.0)	0	(0.0)	3	(1.75)
Sum DR5	14	(41.18)	0	(0.0)	0	(0.0)	27	(15.74)
1301-1305	16	(47.06)	1	(16.67)	1	(12.5)	24	(13.99)
1401-1410	1	(2.94)	0	(0.0)	0	(0.0)	4	(2.33)
0701-0702	3	(8.82)	1	(16.67)	0	(0.0)	34	(19.82)
0801-0805	1	(2.94)	1	(16.67)	1	(12.5)	10	(5.83)
0901	0	(0.0)	0	(0.0)	1	(12.5)	2	(1.17)
1001	2	(5.88)	2	(33.33)	0	(0.0)	2	(1.17)
HLA DQB1*	(n=34)		(n=6)		(n=8)		(n=179)	
0201-0203	4	(11.76)	3	(50.0)	6	(75.0)	75	(41.90)
0401-0402	8	(23.53)	2	(33.33)	0	(0.0)	9	(5.03)
0501-0504	11	(32.35)	2	(33.33)	3	(37.5)	60	(33.52)
0601	0	(0.0)	0	(0.0)	0	(0.0)	1	(0.56)
0602/9-12/14	14	(41.18)	3	(50.0)	1	(12.5)	58	(32.50)
0301-4/11-12	3	(8.82)	1	(16.67)	4	(50.0)	96	(53.63)

When allele phenotype frequencies were compared, a significant increase in HLA-DRB1*0301-0303 was observed in the anti-Ro positive BC SSc patients, [1] p=0.04. No further significant differences were observed in the BC or SAB populations. SAB, South African Black; BC, British Caucasian.

4.5: DISCUSSION

A number of HLA alleles have previously been associated with an increased frequency of SSc and SSc subsets, and associations vary when different populations are examined, particularly when different racial groups are considered. (84;86). A variation in SSc autoantibody specificity is also recognised between different racial origins (24). However, HLA association with the disease is suggested to be independent of ethnic background. To support the hypothesis that HLA plays a significant role in SSc, one might expect that any differences in autoantibody profiles, between races, would be reflected in the purported associated HLA alleles. Thus despite the different frequencies of autoantibodies observed in the different races, HLA and ANA associations would be expected to remain the same. The work in this chapter has investigated autoantibody profiles, HLA and SSc disease subset of two, ethnically different, SSc patient populations, and considers this concept.

In concordance with reports, autoantibodies were present in more than 90% of patients in both populations (BC 92%, SAB 93%). However, the overall autoantibody profiles of the populations were very different. The majority of BC patients had an autoantibody to one of the major serologic groups (ACA, ATA or ARA III) (67%), which was not the case in the SAB population (26%). The majority of SAB sera had an autoantibody to either anti-U3RNP, anti-Ro or an unidentified autoantigen(s). The largest serological group in the SAB patient population was the positive unidentified autoantibody group (22%). Immunoprecipitation and immunodiffusion techniques used to identify autoantibodies, employed known protoype sera derived from a Caucasian SSc patient cohort. SAB patients demonstrated banding patterns on immunoprecipitation that were uncharacteristic to those seen in the BC population, and lines of identity could not be established, using any of the control sera, on immunodiffusion. Thirteen percent of SAB patients had anti-Ro autoantibodies, significantly more than those detected in BC ($p=0.03$), and more than has been previously reported (45). These results demonstrate that not only is the overall SSc autoimmune profile quite different in the two races, but certain autoantibody specificities are apparent in the SAB SSc population that have not been observed in the BC population.

The greatest differences of autoantibody prevalence between the two races were in agreement with previous reports. The most striking difference was in the minor serologic group, anti-U3RNP (BC <1% *vs.* 18% SAB, p<0.0001), an observation that has been reported by a number of groups (24;186;187). Anti-U3RNP has previously been associated with severe SSc (186) although this was not the case in this SAB population. The frequency of ACA also varied greatly. It has previously been reported that the occurrence of ACA is reduced in Blacks compared to Caucasians (23;187). ACA was significantly reduced in the SAB patients compared to the BC SSc population in this study (BC 38.5% *vs.* 9% SAB, p=0.0002). Although absent entirely from one Black population (24), anti-PmScl was detected in one SAB patient, also with an unidentified specificity. Thus, it seems that autoantibodies directed against Pm-Scl are rare in Blacks.

It is evident in this study that the overall HLA background of each ethnic SSc population is quite different. Significant variences in allele frequencies were identified in both ethnic SSc patient populations, compared to their etnically matched control group. Of particular interest was that the observed differences in allele frequencies, were with contrasting alleles, in each of the populations. An increase in frequency of HLA DRB1*0301-0303 and a decrease in HLA DQ6 and DQ3 were observed in the BC population compared with an increase in HLA-DR2, more specifically HLA DRB1*1501-1502, HLA DRB1*1301-1305 and an increase in HLA DQB1*0501-0504 were identified in the SAB population.

HLA associations become more intriguing when autoantibody subgroups are considered. Typically in SSc, ACA has been associated with HLA DR1 and DR4 (63;86;166;188). ACA was significantly associated with HLA-DRB1*0101-0102 and HLA DR4 in the BC population. ACA is regarded as a marker of the limited form of SSc, and indeed ACA positive BC patients showed a significant increase in this form of the disease (p<0.0001). In concordance with this observation, pulmonary fibrosis, a clinical manifestation associated with more severe disease, was observed to be negatively associated with the BC ACA patients. As mentioned, HLA DR1 was increased in lcSSc BC patients, a type which has previously been reported as absent from a group of Japanese SSc patients with diffuse disease (189). Here, the SAB population, which included significantly more patients with dcSSc, showed a reduction

in HLA DR1. In the anti-Ro serological group significant HLA associations with HLA-DRB1*0301-0303 were identified in both the SAB and BC patient populations. Incidentally, these groups were of quite different sizes in each population (BC 4% and SAB 13.5%). Thus overall, the differences in the frequencies of HLA alleles, serological autoantibody and clinical profile, appear related and support the hypothesis that the HLA plays a significant role in SSc.

However, not all associations were equivalent in each population. Although this may seem to weaken the hypothesis, this could arise for a number of reasons. One contributory factor could be the small numbers occurring in some of the autoantibody groups. Also, discordance of associations with disease subtype between the populations could be partially due to differential assignation of disease subtype at different institutions, despite classification criteria, or maybe referral bias. Important data in this respect would be disease duration; A population including mostly patients with early disease, classed as lcSSc, may well be on course to develop dcSSc. In contrast to this a population may comprise patients who have suffered the disease for much longer. Thus those patients destined to develop dcSSc, would have done so. The two populations would, overall, give quite different profiles.

Associations identified in the ATA BC population were with HLA-DRB1*1101-1104 alleles, a subtype of the broad HLA DR5 type, which has frequently been reported in Caucasian and Black races amongst others (83; 190). No HLA associations were observed in the SAB ATA positive population, however this group was rather small (n=6). ATA patients have regularly been reported to have the highest frequency of PF (86; 188) and indeed this was the case in the BC patients. However, SAB patients lacking any identifiable autoantibody were most likely to suffer PF. SAB ATA positive patients also showed no association with disease subtype, although conversely a weak association with dcSSc was identified in the BC ATA positive patients (p=0.08).

Studies by Kuwana *et al.* ('99) and Cram *et al.* ('93) investigated immune reactivity to different portions of recombinant topo-I protein (190;191). Both groups concluded that different races characteristically recognised different portions of the protein. The same portion, (adjacent to the carboxyl terminus), was recognised in White and Black SSc patients (190). It would be interesting to consider whether patients recognising different

portions of this autoantigen could be further defined clinically and/or genetically. A study by Greidinger *et al.* suggested that autoantibodies to topoisomerase I but not ARA II were associated with lung function. (Although the report does not state whether ATA was found accompanying RNAP II or RNAP III or either) (192). When patients in the ATA serological group in this study were further divided according to other autoantibodies present in the serum, associations with pulmonary fibrosis or disease subtype could not be further defined. Although no HLA associations were identified in the ARA III group, in agreement with previous reports an association with dcSSc was observed (188;193).

Although differences in the autoimmune response between races have not been defined, it is evident that HLA background, autoantibody profile and clinical course are ethnically variable. Exactly how genetic status, immunological and clinical processes relate to, or are independent of each other, is yet to be established, but results here suggest that associations between them are independent of ethnicity. Equivalent mutual exclusivity of autoantibodies was observed in both patient populations and was generally in concordance with reports, despite autoantibody multi-specificity in the majority of serum. This further suggests that the immune response is not only predetermined, but characterised by a factor or factors other than ethnicity, and implies a significant role of the HLA. Small populations with a high incidence of scleroderma, such as the Oklahoma Choctaw (31), provide valuable models for further investigating this notion. These populations have restricted gene pools, which not only support the idea of a genetic component to the disease, but also provide an exceptional opportunity for extensive genetic analysis.

HLA studies have revealed distinct evolutionary patterns for the HLA loci in ethnically diverse samples resulting in different predominant HLA haplotypes (87). It has been suggested that immunogenetic factors, including HLA class II molecules, probably mediate SSc-related autoantibody production (24). In this light, the variation in the prevalence of SSc disease subtype observed in different populations, coupled with variation in autoantibody frequencies, supports the hypothesis that the HLA plays a role in SSc. Observations here indicate that the production of SSc related serum ANA is related to immunogenetic background, as has also been recognised in other studies (24).

CHAPTER 5

CLINICAL FEATURES AND MHC CLASS II ASSOCIATIONS IN INDIVIDUALS WITH ANTICENTROMERE ANTIBODIES

5.1: INTRODUCTION

Anticentromere antibodies (ACA) are most commonly identified in patients with SSc, but may also be present in a number of other autoimmune rheumatic conditions including rheumatoid arthritis (RA), SLE and primary Sjogren's syndrome. In a study of patients with ACA, attending a rheumatology outpatient clinic, Vlachoyiannopoulos *et al.* found that 64% of patients with ACA had lcSSc, 17% with primary Sjogrens syndrome and 12% with idiopathic Raynaud's phenomenon (194). In a similar study, Chan *et al.* found that the majority of their patients with ACA had either the limited form of scleroderma (described in the report as CREST) (55%) or Raynaud's phenomenon (29.6%) (195).

In scleroderma, the presence of ACA represents one of the three, recognised, major autoantibody groups. Approximately 26% of scleroderma patients are reported to have ACA (45), however this figure is lower in some non-Caucasian populations, including American Blacks (23), and Thai patients (196). SSc patients with ACA are most likely to have lcSSc. Of these patients most will have Raynaud's phenomenon and an absence of renal involvement. Patients with lcSSc and ACA are also thought to have an increased risk of major peripheral vascular occlusive disease (60). As discussed earlier, Raynaud's phenomenon may be the first manifestation of SSc and the presence of ACA in patients with Raynaud's phenomenon identifies those patients at risk of going on and developing other features of lcSSc. The early identification of autoantibodies in these patients could bring attention to potential cases of SSc (47).

ACA are reported to be present in 10-20% patients with primary biliary cirrhosis (PBC), a condition often found in overlap with SSc (57). PBC patients who have ACA often have some features of SSc as well, such as Raynaud's phenomenon and sclerodactyly (197). ACA has also been identified in patients not only with rheumatoid arthritis (RA), but in patients with RA concurrent with SSc (198). The latter study detailed three case reports of ACA positive RA patients who proceeded to develop lcSSc, 11-50 years after the onset of RA (198). It has been suggested that the presence of deforming arthritis, co-occurring with SSc may represent a distinct, unique overlap condition (199). ACA has been identified as a significant risk factor for cancers in some patients with SSc (127). Some patients with SLE may have ACA. An increase in the age of disease onset and, an

increased occurrence of Raynaud's phenomenon, has been identified in ACA positive SLE patients compared with ACA negative SLE patients (200). The number of SLE patients with ACA has recently been suggested to be higher than first appreciated and may identify a distinct SLE group (200). Some Sjogren's syndrome patients may also have ACA. Analysis of a population of primary Sjogren's patients, from an Italian institution, identified just over 16% of patients with ACA (201).

Genetic associations with ACA have previously been reported and mainly refer to those patients with SSc. Genth *et al.* reported that SSc patients with ACA were frequently positive for the broad phenotypes HLA DR1, DR4 or DRw8 (80). These observations were supported by Briggs *et al.*, who reported a significant association of the presence of either DR1 or DR4 with the expression of ACA (107), and similarly by Reveille *et al.* with regard, more specifically, to HLA DRB1*0403 and 0407, and HLA DR5 (202). Association of ACA in SSc patients with the HLA DQB1 region have also been reported. Analyses has revealed a significant increase in frequency of HLA DQB1*0501 (81;86), and a decrease in DQB1*0201 in ACA positive SSc patients (81). Further analysis of HLA DQB1 has identified genotype sequences common to ACA positive SSc patients. A polar glycine or tyrosine residue at position 26 of the DQB1 first domain (HLA-DQB1: 26:L) was present in all ACA positive SSc patients in a study by Reveille *et al.*, implying the necessity for a non-HLA-DQB1: 26:L allele for the generation of autoantibody (202). However this observation was not supported in study of Japanese, ACA positive, SSc patients (86).

Since the first description of autoantibodies directed toward the centromere region of cells in 1980, it has become recognised that a number of distinct protein components may be targeted, as described in the main introduction to this work (chapter 1). As mentioned, the three major centromere antigens CENP-A (19Kd), CENP-B (80Kd) and CENP-C (140Kd) are often targeted, whilst antibodies to CENP's D, E, F and G occur less often. CENP-B is the most commonly recognised CENP antigen (203). CENP-A and CENP-B are recognised by IgG antibodies, and CENP-C by IgM antibodies (163). Various antigen regions (epitopes) on the CENPs have been identified. At least five epitopes have been identified on CENP-B, one of which is shared by CENP-A and CENP-C (57), and nine epitopes have been reported for CENP-C (204).

There have been very few investigations into the characterisation of the ACA response, with regard to the presence of specific anti-CENPS and associations with particular clinical features or clinical profile. Some trends have been observed regarding the major autoantigens (CENP A, B and C), but not with the less frequently occurring anti-CENP autoantibodies. When a comparison between patients with anti-CENP-A, B, and C was made with anti-CENP-E positive patients, no unique clinical features in the anti-CENP-E positive group were identified (205). A study by Vazquez-Abad and colleagues investigated the autoantibody specificity of patients without Raynaud's phenomenon or SSc (group A), compared to patients with these conditions (group B). It was concluded that the ACA specificity between the two groups was different. Most obviously, far fewer group A patients recognised CENP B, compared to group B patients. Moreover, group A patients who did recognise CENP B were thought to bind different epitopes to those recognised by patients in group B (206). Interestingly a study investigating reactivity's toward CENP A and/or CENP B antigen revealed a significant difference in the distribution of the two autoantibody-specificities in SSc patients compared to an RA and SLE patient population. Ninety seven percent of SSc patients showed reactivity with both CENPs A and B, where as 83% of RA and SLE ACA positive samples showed mutually exclusive reactivity's with antibodies directed against either CENP A *or* CENP B (207). Further, similar, comparisons between ACA positive patients with regard to the combination of CENPs recognised may further identify clinical subgroups of disease.

5.2: CHAPTER AIMS

The objectives of the work in this chapter were; to characterise the clinical, immunogenetic and autoantigenic profile of ACA positive patients, and to assess the clinical course and disease evolution represented by an ACA positive population. This was investigated by means of the following aims:

I. To assess the clinical profile of ACA positive patients attending a rheumatology clinic

II. To identify any genetic associations with disease, in patients with ACA

III. To identify any associations of HLA-DRB1 and/or DQB1 alleles, (at the allele level or with previously reported amino acids at particular loci), with ACA positivity.

IV. To analyse disease outcome in a subgroup of ACA positive patients with a follow up of four years or more

V. To analyse specific polypeptide antigens (CENPs) recognised by ACA positive sera and to identify associations with disease or HLA background

5.3: MATERIALS AND METHODS

Patients.

Two populations of patients, selected from two institutions, were included in this part of the study:

Fifty-five patients attending the Royal National Hospital for Rheumatic Diseases (RNHRD), who tested positive for serum ACA, comprised the 'first data set'. Of these patients, 43 had clinical data available and were included in the clinical profile study, 30 in the HLA analysis and 25 in the longitudinal study.

A further 47 patients, attending a rheumatology clinic in Manchester were identified as ACA positive, and formed the 'second data set'. Of these patients 41 were included in the HLA analysis section.

Detection of ACA autoantibody

The presence of ACA was established by indirect immunofluorescence of patient sera on Hep-2 cell substrate (method 2.4.2). ACA was assigned when the characteristic fluorescent pattern of discrete dots, (in interphase cells), and fluorescence of a metaphase plate (in dividing cells) was identified.

HLA-DRB1 and DQB1 typing

HLA-DRB1 and DQB1 alleles were identified by PCR-SSP genetic typing, according to method 2.2.2.

Identification of CENPs

Analysis of autoantigenic polypeptide recognition was performed on 44 ACA positive patients. Identification of CENPs recognised by ACA positive sera was investigated by immunoblotting, as described in method 2.4.5.

Clinical profile

Case notes of 43 patients, (all from the first data set), who had been found ACA positive during the period of July 1992 and June 1997, (five years), were reviewed and the precise clinical diagnosis at the time of first presentation was documented.

Longitudinal disease profile

Follow up of at least 4 years was available on 25 ACA positive patients. Disease evolution, if any, over the total period of follow up available was documented. If a patient had missed an appointment, their GP was contacted and details on the latest follow up were obtained. This was carried out with, and under the guidance of, a rheumatology clinician.

Statistical analysis

Statistical analysis was carried out according to that described in the main materials and methods.

5.4: RESULTS

5.4.1: Identification of ACA positive patients attending a connective tissue disease clinic at one of two selected institutions

Identification of ACA

Fifty-five patients attending the RNHRD were identified as ACA positive by indirect immunofluorescence and accepted into the study (data set one). Figure 4.2 is an example of a typical fluorescence pattern seen from ACA positive sera. Of a total 127 patient sera, collected from patients attending the rheumatology clinic at the Hope hospital, Salford, 47 (36.5%) were found to have ACA present in their serum (data set two).

5.4.2: Assessment of the clinical profile represented by an ACA positive patient population attending a CTD clinic, subsequent identification of any genetic associations with disease

The following includes patients from the first data set only.

Patients

A total of 55 patients, who attended the RNHRD between July 1992 and June 1997 were identified as ACA positive. Clinical information was available on 43 of these patients from which the overall ACA clinical profile was assessed. This included 41 female: 2 male with a median age of 59 years (range 32-87 years).

Assessment of clinical profile represented by ACA patients received through a CTD clinic

Thirty-one, (72%) patients were diagnosed with lcSSc, of which 26 (60.5%) had lcSSc alone and six had a form of arthritis as well (table 5.1). Four patients were diagnosed with Sjogren's syndrome, of which one patient also had an overlap with SLE and arthritis. Five patients had erosive RA, (3 seropositive, 2 seronegative). One of the patients with seronegative erosive RA also had fibromyalgia and Hashimoto's thyroiditis. One patient was diagnosed with UCTD (table 5.1).

Positive predictive value of ACA for lcSSc

The positive predictive value of ACA for lcSSc was 72% (table 5.2). The next most frequent association of ACA was with arthritis (28%).

5.4.3: Associations of ACA with HLA-DRB1 and/or DQB1 alleles

HLA-DRB1 and DQB1 phenotype frequencies were analysed and compared to the phenotype frequencies of 250 normal controls.

ACA associations with HLA: Data set one.

DNA was available from 30 ACA positive patients for HLA typing. HLA phenotype frequencies of these patients are shown in table 5.3 and are compared to 250 normal controls. HLA-DRB1*0101-0103 was significantly increased in the patients compared to controls (patients 40% *vs.* 21% controls, p=0.02). A decrease in HLA-DRB1*1301-1305 was also noted in patients however this did not reach significance (patients 7% *vs.* 21.5% controls, p=0.09).

In the HLA-DQB1 alleles one significant difference was apparent between patients and controls. HLA-DQB1*0501 was significantly raised in patients (patients 41% *vs.* 30% controls, p=0.01) (table 5.3).

ACA associations with HLA: Data set two.

HLA typing was performed on 44 patients from the second data set and several significant differences in phenotype frequencies were observed when they were compared to normal controls. HLA-DRB1*0101-0103 was significantly increased in ACA patients (patients 44% *vs.* 21% controls, p=0.001). HLA DR5 appeared decreased in patients (patients 5% *vs.* 19% controls, p=0.05), however when HLA-DR5 subtypes were analysed any significance was lost. A slight, notable, decrease remained in the DRB1*1101-1104 allele (patients 5% *vs.* 16% controls, p=0.09).

One significant difference was observed in the HLA-DQB1 alleles. HLA-DQB1*0501 was significantly increased in patients compared to normal controls (patients 43% *vs.* 21% controls, p=0.001) (table 5.3).

ACA associations with HLA: data sets one and two combined

To try and reduce the chance of false associations due to small numbers, the data sets were combined and the analysis repeated. Particular attention was paid to see if any previous associations were still apparent, or any trends had reached significance.

HLA-DRB1*0101-0103 was significantly raised in patients compared to controls (patients 42% *vs.* 21% controls, p=0.0003). A significant reduction in DR5 alleles was also observed (p=0.03). When this broad type was broken down HLA-DRB1*1101-1104 remained significantly decreased in patients compared to the controls (patients 5.5% vs. 19% controls, p=0.03). HLA-DRB1*0401-0411 was significantly raised in patients when compared to controls (patients 48% *vs.* 35% controls, p=0.050, and HLA-DRB1*0701-0702 was significantly decreased (patients 14% *vs.* 25% controls, p=0.04). Of note, a significant number of ACA patients were found to be HLA-DRB1*0101-0102 and/or HLA-DRB1*0401-0411 positive when compared to controls (patients 73% *vs.* 51.5% controls, p=0.001; p=0.01 with $B._{Corr.}$). (Table 5.4).

HLA-DQB1*0501 was significantly increased in patients compared to controls (42.5% *vs.* 29% patients, p=0.0002).

Analysis of ACA and HLA-DQB1 position 26

The data was examined for the absence of the amino acid leucine at position 26 of the HLA-DQB1 third domain (HLA-DQB1: L: 26). The alleles HLA DQB1*0501, 0502, 0503, 0402, 0601, 0301 all lack leucine at position 26 of the first domain.

Data set one

HLA-DQB1 position 26 was analysed in 29 patients in data set one. Five patients (17%) lacked leucine at position 26 from both alleles. Of the remaining patients 13 (45%) were heterozygous and 11 (38%) were homozygous for HLA-DQB1: L: 26. When these figures were compared to normal controls there were no significant differences.

Data set two

In the second data set, (n=41), ten (25%) patients lacked leucine from position 26 of both HLA-DQB1 alleles. Twenty-four (58.5%) of patients were HLA-DQB1: L: 26 heterozygous, which was significantly less than the number of heterozygous normal

controls (p=0.03). Seven (17%) patients were homozygous for leucine at this locus. This was also a significantly lower number of individuals compared to normal controls (p=0.01).

Data sets one and two combined.

When the two data sets were combined 15 (21.5%) patients lacked leucine from position 26 of both HLA-DQB1 alleles. 37 (53%) were heterozygous for leucine at this position. Eighteen (25.5%) patients were homozygous for HLA-DQB1: L: 26, which was significantly lower when compared to normal controls (p=0.01). Significantly more ACA patients lacked at least one allele coding for leucine at this position compared to controls (patients 52/70 (74.2%) *vs.* 144/250 (57.6%) controls, p=0.01).

5.4.4: Analysis of the longitudinal profile of a subgroup of patients with a follow up of four years or more

Patients

ACA patients with a follow up of at least four years were evaluated to assess longitudinal profile. Twenty-five patients (24 female: 1 male) were included in this particular investigation with a mean follow up of 59 months (range: 48-144 months). The average age of initial diagnosis was 56 years.

Initial diagnosis and longitudinal profile

LcSSc

The initial diagnosis, associated or other conditions suffered, and details of follow up, can be seen in table 5.5. Sixty eight percent of patients, (17/25), had an initial major diagnosis of lcSSc of which four individuals initially presented with Sicca syndrome. Fourteen of these patients maintained an overall stable course. One showed slight skin progression, two had troublesome Raynaud's, and one developed Sicca syndrome during the course of study. Of the remaining 3 patients in this group one showed significant disease progression and also developed osteoporosis (patient PP). One patient developed scleroderma renal crisis at 54/75 months of follow up. This occurred after a stable and mild course of about four years. The patient developed irreversible

renal failure despite early and aggressive treatment, which included ACE inhibitors. The third patient died of a cause unrelated to SSc (myocardial infarction), at 134 months of follow up.

LcSSc and erosive rheumatoid arthritis

Three patients were initially diagnosed with lcSSc and erosive RA. Two of these patients were being treated with methotrexate (/required disease modifying therapy). There was no significant progression of scleroderma in these patients.

Seronegative/positive erosive rheumatoid arthritis

One patient presented initially with seronegative erosive RA and sicca syndrome, and one with seropositive erosive RA who also had mononeuritis multiplex, gangrene and Sicca syndrome. Neither patient showed signs of developing scleroderma over the duration studied (48 and 56 months respectively).

Other major initial diagnosis

One patient who was initially diagnosed with Raynaud's phenomenon and oligoarthritis showed no disease progression. One patient with UCTD showed no change over the 54 months studied.

5.4.5: Analysis of specific polypeptide antigens (CENPs) recognised by ACA positive sera and identification of associations with disease or HLA background

Forty-four ACA patients comprising data sets one and two were immunoblotted according to method 2.4.5. The aim of the work was to identify the specific CENP proteins recognised by an individuals' serum. An analysis would then have been carried out to identify any associations of CENP proteins recognised with disease profile and/or HLA background. This was the last part of the laboratory research carried out for this project and unfortunately there were problems with the technique and thus there was not sufficient time to obtain the anticipated results. However some deductions can be made from the results that were achieved, although, it must be stressed that this is regarded as unfinished work. Immunoblotting work on some of the ACA positive patients had been previously carried out at Bath, as part of another study, and this work will be considered

in the discussion of this chapter with regard to the HLA typing.

Immunoblotting of ACA positive patients

Forty-four ACA positive patients were immunoblotted. It was established that 41 (93%) of these patients possessed autoantibody recognising CENP A. No further conclusions with regard to the other CENPs could be made from this work due to reasons explained.

Table 5.1: Disease affecting ACA positive patients

Disease Phenotype	No. Patients (n=43)
LcSSc	26
LcSSc/ Erosive RA	1
LcSSc/ Erosive polyarthritis	1
Lssc/ Polyarthritis	1
LcSSc/ Seropositive erosive RA	1
LcSSc/ RA	1
Primary Sjogren's syndrome	2
RA, Sjogren's syndrome	1
Raynaud's Phenomenon/Arthritis	1
SLE	1
SLE/ Sjogren's syndrome	1
Seronegative erosive RA	1
Seronegative erosive RA, Fibromyalgia, Hashimotos thyroiditis	1
Seropositive erosive RA	3
UCTD	1

Disease phenotypes encountered in an ACA positive patient population attending a connective tissue disease clinic.

Table 5.2: Spectrum of diseases affecting ACA positive patients

Disease	No. Patients (n=43)	(%)
LcSSc	31	(72)
Arthritis	12	(28)
Sjogren's syndrome	4	(9)
Raynaud's phenomenon (In absence of SSc)	1	(2.5)
SLE	2	(5)
UCTD	1	(2.5)

The prevalence of each of the diseases affecting the ACA positive patients is shown.

Table 5.3: HLA phenotype frequencies of two ACA patient populations compared to normal controls

Allele	Data set 1		Controls		Data set 2	
HLA DRB1*	(*n*=30)	(%)	(*n*=250)	(%)	(*n*=41)	(%)
0101-0103	12[1]	(40.0)	53	(21.2)	18[2]	(44.0)
1501-1502	6	(20.0)	65	(26.0)	7	(17.1)
0101-1602	0	(0.0)	6	(2.4)	0	(0.0)
Sum DR2	6	(20.0)	71	(28.4)	7	(17.1)
0301-0303	8	(26.7)	53	(21.2)	6	(14.6)
0401-0411	14	(46.7)	88	(35.2)	20	(48.8)
1101-1104	2	(6.7)	41	(16.4)	2	(4.9)
1201-1202	1	(3.3)	7	(2.8)	0	(0.0)
Sum DR5	3	(10.0)	46	(19.2)	2[3]	(4.9)
1301-1305	2	(6.7)	54	(21.5)	9	(22.0)
1401-1410	0	(0.0)	5	(2.0)	1	(2.4)
0701-0702	5	(16.7)	63	(25.2)	5	(12.2)
0801-0805	2	(6.7)	13	(5.2)	5	(12.2)
0901	0	(0.0)	4	(1.6)	0	(0.0)
1001	0	(0.0)	2	(0.8)	1	(2.4)
HLA DQB1*	(n=29)		(250)		(n=44)	
0201	12	(41.0)	88	(35.2)	12	(27.3)
0401	0	(0.0)	0	(0.0)	0	(0.0)
0402	0	(0.0)	10	(4.0)	6	(13.6)
0501	12[4]	(41.0)	52	(28.8)	19[5]	(43.2)
0502	0	(0.0)	9	(3.6)	0	(0.0)
0503	0	(0.0)	5	(2.0)	2	(4.5)
0601	1	(3.4)	3	(1.2)	0	(0.0)
0602	6	(20.7)	60	(24.0)	7	(16.0)
0603/8	1	(3.4)	27	(10.8)	2	(4.5)
0604	1	(3.4)	28	(11.2)	4	(9.1)
0301	7	(24.1)	91	(36.4)	16	(36.4)
0302	9	(31.0)	51	(20.4)	13	(29.5)
0303	1	(3.4)	31	(12.4)	3	(6.8)

Significant differences in HLA allele frequencies were observed in both data sets when they were compared to normal controls, p values with OR(CI) are shown. HLA-DRB1*0101-0102 was raised in data set 1 and 2 ([1]p=0.02: 2.4(1.1-1.5) and [2]p=0.0017: 2.9(1.5-5.7), respectively). Other significant differences observed are as follows; [3]p=0.05: .22(0.05-0.97), [4]p=0.01: 2.5(1.2-5.6), [5]p=0.001: 3.3(1.7-6.5).

Table 5.4: HLA phenotype frequencies of data sets one and two combined, compared to normal controls

Allele	Data sets 1 & 2 combined		Controls	
HLA DRB1*	(n=71)	(%)	(n=250)	(%)
0101-0103	30[1]	(42.3)	53	(21.2)
1501-1502	13	(18.3)	65	(26.0)
101-1602	0	(0.0)	6	(2.4)
Sum DR2	13	(18.3)	71	(28.4)
0301-0303	14	(19.7)	53	(21.2)
0401-0411	34[2]	(47.9)	88	(35.2)
1101-1104	4[3]	(5.6)	41	(16.4)
1201-1202	1	(1.4)	7	(2.8)
Sum DR5	5[4]	(7.0)	46	(18.4)
1301-1305	11	(15.5)	54	(21.5)
1401-1410	1	(1.4)	5	(2.0)
0701-0702	10[5]	(14.1)	63	(25.2)
0801-0805	7	(9.9)	13	(5.2)
0901	0	(0.0)	4	(1.6)
1001	1	(1.4)	2	(0.8)
HLA DQB1*	(n=73)		(n=250)	
0201	24	(32.9)	88	(35.2)
0401	0	(0.0)	0	(0.0)
0402	6	(8.2)	10	(4.0)
0501	31[6]	(42.5)	52	(28.8)
0502	0	(0.0)	9	(3.6)
0503	2	(2.7)	5	(2.0)
0601	1	(1.4)	3	(1.2)
0602	13	(17.8)	60	(24.0)
0603/8	3	(4.1)	27	(10.8)
0604	5	(6.8)	28	(11.2)
0301	23	(31.5)	91	(36.4)
0302	22	(30.1)	51	(20.4)
0303	4	(5.5)	31	(12.4)

Several significant differences in phenotype frequencies were apparent when both data sets were combined and compared to normal controls; p-values with OR(CI) are as follows: [1]p=0.0003: 2.7(1.6-4.8), [2]p=0.05: 1.7(1.0-2.9), [3,4]p=0.03: 0.3(0.1-0.9), [5]p=0.04: 0.5(0.2-1.0), [6]p=0.0002: 2.9(1.7-5.2).

Table 5.5: Longitudinal clinical profile of ACA positive patients with a follow up of greater than four years

Patient ID.	Sex	Age at diagnosis	Initial Diagnosis (Major clinical manifestation)	Consensus of follow up and comments	Total follow-up time (months)	Associated/ other conditions
EC	F	69	Seronegative erosive RA	No SSc	048	Sicca Syndrome
SH	F	62	LcSSc	Slight skin progression	049	
JL	F	56	LcSSc	No Progression	049	Sicca syndrome
BD	F	36	LcSSc	LcSSc	050	
AL	F	52	LcSSc	No Progression	051	
PP	M	65	LcSSc	Significant Progression, (developed osteoporosis)	053	Osteoporosis
BR	F	59	LcSSc	No Progression	054	
JR	F	35	UCTD	UCTD	054	
CD	F	73	LcSSc	No Progression	055	
BM	F	62	Raynauds/ oligoarthritis	No Progression	056	
PB	F	46	Seropositive erosive RA	One visit only. Still no SSc to date.	056	Mononeuritis multiplex, gangrene, sicca syndrome
RM	F	59	LcSSc	No Progression	059	Sicca syndrome
LO	F	72	LcSSc, Erosive polyarthritis	No Progression, feeling better.	063	
DH	F	48	LcSSc	LcSSc. Troublesome Raynaud's	074	
PD	F	45	LcSSc	Developed SSc renal crisis at 66 months FU.	075	None
JM	F	42	LcSSc	LcSSc. Raynaud's quite severe	079	
LP	F	42	LcSSc	No Progression	082	None
HS	F	61	LcSSc	No Progression. Chest infections	082	Sicca Syndrome

Con't.

DH	F	56	LcSSc	No Progression	102	
PaD	F	33	Raynaud's	Ltd. SSc	113	
IO	F	61	LcSSc, Seropositive erosive RA	Ltd. SSc -no progression. Aggressive RA - MTX	113	SPERA
OA	F	52	LcSSc	Developed Sicca syndrome	130	PBC, Sicca syndrome
ML	F	80	LcSSc	Died '95 of MI and pneumonia	134	
LD	F	72	LcSSc, Erosive RA	Severe Raynaud's; digital ulcers. MTX for polyarthritis. LcSSc stable.	144	RA and Sicca syndrome

ACA patients with ≥4 years follow up (mean 59 months). Details of the initial diagnosis made are shown. Associated and/or other conditions regarded as significant have also been noted, and also comments with regard to disease progression or development over the follow up period. The majority of patients had an initial diagnosis of lcSSc (68%), and most maintained an overall stable course.

5.5: DISCUSSION

Autoantibodies directed against the centromere have been reported in a number of rheumatic conditions, and the ACA positive population identified in this study encompasses many of them. A total of 102 patients, with a median age of 59 years, were found to be positive for ACA. All, except two patients, were female. ACA is infrequently detected in male patients (194;208). The majority, 72%, of ACA positive patients had lcSSc. Previous studies also report the preponderance of lcSSc in ACA positive individuals (194;209-211). With the exception of a few reported cases, ACA is extremely uncommon in dcSSc patients (194), and in concordance with such reports no dcSSc patients were identified in the present ACA patient population. ACA was also moderately predictive for arthritis and identified 28% of patients. Overall, ninety-one percent of ACA positive patients had a diagnosis of lcSSc and/or a form of arthritis. Of note, the arthritis in many of the patients was erosive. Of the remaining patients who did not fall into the SSc/arthritis category, two (7%) had Sjogren's syndrome and one had UCTD. This observation lends support to the report by Tubach *et al.*, who in a study of ACA positive individuals without Scleroderma or Raynaud's phenomenon identified Sjogren's syndrome as the most common diagnosis (209).

The results from the longitudinal study suggest that the prevailing impression of ACA delineating a subset of SSc patients, which have a stable and mild course, should be regarded with some caution. Signs of deterioration or disease progression were apparent in as many as seven (28%) patients in the longitudinal study. During the course of follow up, one patient (paD), developed lcSSc, following an initial diagnosis of Raynaud's. This example demonstrates the significance of identifying ACA in Raynaud's patients whose disease may be likely to evolve to lcSSc (47;212). The most extreme case of deterioration was a patient who developed scleroderma renal crisis, culminating irreversible renal failure. The complication occurred suddenly after a seemingly stable course of about four years. This observation suggests that even ACA positive SSc patients with mild disease should receive regular follow-up. Further, it must be appreciated that a minority of ACA positive patients may have unfavourable outcome: as is evident in this study, and as others have reported (210;213).

The co-occurrence of lcSSc and a form of arthritis, which was described in three

patients by Zimmerman *et al.*, was identified in five patients in this study (198). Misra *et al.* suggested that ACA positive patients with both lcSSc and arthritis might represent a distinct, unique overlap condition (199). Patients in this study support this hypothesis clinically however genetically there were no striking observations. Overall, HLA DR4 and DR1 were significantly raised in the ACA patient population when compared to the normal control population. As mentioned in the main introduction to this thesis, specific DR4, and DR1, alleles carry the 'shared epitope' motif associated with rheumatoid arthritis (73). Only one lcSSc/arthritis patient was HLA DR4 positive, and sub-typing revealed an allele that does not bear the mentioned shared epitope (DRB1*0407). However the two remaining patients, from which genetic material was available, both possessed alleles which bear the shared epitope, HLA DRB1*0101-0102. Interestingly, in a report describing ATA positive scleroderma-rheumatoid arthritis patients, all patients were HLA-DR4 positive, however the study does not detail the precise HLA DR4 alleles concerned (214).

Several significant HLA associations were identified in the ACA positive patients. Significant differences in allele frequency were observed when the data sets were considered individually and some became stronger when the two sets were combined. Other significant frequency differences only became apparent when the larger group was considered. An increased frequency in HLA DRB1*0101-0103 in the ACA positive patients compared to the controls was observed when the data sets were considered separately and when they were combined. HLA DR4 was raised in data sets one and two compared to controls, and the difference in frequency became significant when the data sets were combined. These observations are in agreement with previous reports, which have described an association of ACA with the presence of HLA DR1 or DR4 (80;107). A decrease in HLA DRB1*1101-1104 was noticed initially in data set 2, and a significant trend was seen when the two data sets were combined, and compared to controls. In accordance with a report by Morel *et al.*, ACA positive patients demonstrated a significant increase in HLA-DQB1*0501, (in data sets one, two and the combined set) (81).

The most striking observation was the prevalence of at least one HLA DRB1*0101-0102 and/or DRB1*0401-0411 allele in the ACA patients. This was evident in nearly 75% of patients, a significantly greater proportion compared to the control group. The

increased frequencies of these alleles appeared to be reflected in the frequencies of HLA DR7 and DR5, which were significantly decreased in the patients compared to the controls. Thus, it may be interpreted from the results that HLA DR1 and/or DR4 may confer susceptibility for an ACA immune response in some individuals. HLA DQB1*0501 is in linkage disequilibrium with HLA DR1, which may explain the significantly increased frequency observed in this allele.

The presence of at least one HLA DQB1 allele not coding for leucine at position 26 of the first domain has been suspected to be a key requirement for the generation of ACA (202). As position 26 is located in the floor of the antigen-binding cleft, it will directly affect which peptides bind to DQB1. Further, the polarity of the amino acids at this position will affect which peptides are preferentially bound. Reveille *et al.* and McHugh *et al.* reported the absence of leucine at position 26 from at least one allele in 100% of ACA positive individuals studied, implying that the presence of at least one polar amino acid at this locus is essential for an ACA immune response (122;202), respectively. Analysis of this locus in the present study revealed a significant increase in the number of patients who possessed at least one allele not coding for leucine (patients 74% *vs.* 57.5% controls). However, 26% did not bear a polar residue at this locus and thus cannot 'fully' support the idea proposed by the aforesaid.

Some studies have been unable to identify such HLA associations (81;86), which has led them to investigate other loci (81). In accordance with the present study, Morel *et al.* identified a positive association of ACA with HLA DQB1*0501, in SSc patients (81). This allele was present in 47% of individuals, a comparable figure to the number of ACA positive SSc patients positive for this allele in the present study (43%). However, as more than 50% of patients were negative for this allele they reasoned that this association was not strong, and thus analysed DQB1*0501 negative patients in order to determine whether any other associations were playing a role in this form of the disease. Morel *et al.* went on to identify an increased frequency of the DQß1 amino acid sequence TRAELDT (amino acids 71-77) in DQB1*0501 negative, ACA positive SSc patients, which was independent of DQB1*0501 (86). They suggest from their findings that SSc with ACA may be further subdivided into two forms, including those patients with DQB1*0501 and those with DQB1:TRAELDT:71-77 (86). The equivalent analysis of the patients included in this study could not support the associations identified by

The immunoblotting of ACA positive sera for the identification of CENP specificity was, as discussed earlier, incomplete work and therefore largely inconclusive. However analysis of immunoblotting data from 39 ACA positive patients, which had been generated previously by a final year project student at this institution (Lorna Daly), showed reactivity to CENPS A, B and C in 28 patients. The remaining patients showed reactivity to 2 or less CENPs. HLA analysis did not reveal any associations with regard to the CENPs, or combination of CENPs, recognised.

The work in this chapter has identified ACA positive individuals, and investigated the clinical and immunogenetic aspects of the patient population. An association of ACA has been identified with lcSSc, which may show progression in a small number of patients. Identification of ACA also shows a degree of predictability for other rheumatic conditions especially arthritis, which has a tendency to be erosive in these individuals. The results confirm associations of ACA with HLA DR1 and DR4, however associations with specific loci remain modest. Further HLA analysis in larger groups of ACA positive SSc patients may reveal additional disease subtypes.

CHAPTER 6

INVESTIGATION OF GENES INVOLVED IN FIBROSIS: THEIR ASSOCIATION WITH SYSTEMIC SCLEROSIS AND SPECIFIC AUTOANTIBODIES

6.1: INTRODUCTION

As discussed in the main introduction to this thesis, the precise aetiology of SSc is unknown however there is mounting evidence from genetic studies of associations between SSc and polymorphisms in a number of genes that support the hypothesis of a genetic contribution to the disease.

Although SSc is a heterogeneous condition the recognised hallmark of the disease is fibrosis. As previously discussed in chapter 1, fibrotic lesions of scleroderma result from the excess deposition of normal components of connective tissue by a subset of activated fibroblasts. As the activated phenotype of the SSc-fibroblast is lost *in vitro* it is considered that *local* factors are responsible for their original activation (4).

The work in the chapter investigates the genes for proteins implicated in the SSc-fibrotic response. Five candidate genes, which are considered key in SSc-fibrosis have been investigated; TGFβ1, TGFβ2, TGFβ3, B chain of platelet derived growth factor, PDGFB, tissue inhibitor of metalloproteinase-1, TIMP-1 and the α-2 subunit of collagen type V, Col5A2.

There is evidence indicating a role for TGFβ in fibrosis. TGFβ is the primary fibrogenic cytokine, activating and stimulating normal fibroblasts to produce connective tissue components. This cytokine has three structurally and functionally similar isoforms, TGFβ1, TGFβ2 and TGFβ3, which in mammals are each encoded for by separate genes. Once activated from its latent form, TGFβ elicits multiple cellular responses in different cell types that are mediated through high affinity receptors. Activated TGFβ can also associate with TGFβ latency-associated peptide, which prevents its biologic activity (215). Recent studies have supported the role of TGFβ in fibrosis. It has been shown that subcutaneous injection of TGFβ into animals causes rapid fibrosis at the local site (216). An increase in fibroblast collagen synthesis is a recognised response to TGFβ. Plasma TGFβ levels have been reported to be elevated in patients with limited and diffuse SSc (217) and all three TGFβ isotypes have been reported to be up regulated in the inflammatory stage of patients with SSc (218).

TGFβ can induce fibroblasts to produce their own TGFβ, in an autocrine fashion, in addition to promoting the production of other pro-fibrotic cytokines such as platelet-derived growth factor (PDGF). TGFβ can also affect the pathways for ECM degradation; the factors which degrade ECM (plasminogen activator and MMPs) are inhibited by TGFβ, whilst those factors which inhibit the activity of degrading molecules (plasminogen activator inhibitor and TIMPS) are stimulated (215;219).

Platelet derived growth factor (PDGF) is a 30 KDa protein consisting of two related peptide chains, A and B. This potent fibroblast mitogen exists as three isoforms, PDGF AA, PDGF BB and PDGF AB, all of which are biologically active. PDGF is stored in the α granules of platelets from which it is released after platelet activation. Other cells may also synthesise PDGF including macrophages and endothelial cells. At low concentrations PDGF is a chemoattractant for fibroblasts. It is chemotactic and an activator for monocytes and neutrophils and a potent modulator for T cell functions. (For review see http://www.copewithcytokines.de/cope.cgi?004931).

PDGF has been implicated in the pathogenesis of fibroproliferative disorders. A role for the factor in fibrosis is indicated by the impairment of wound healing in the presence of antibodies to PDGF (40). The production of collagens and other ECM components may be upregulated by PDGF (220). It also enhances the production of IL-6, a profibrotic cytokine, from SSc fibroblasts (221).

Tissue inhibitors of metalloproteinase (TIMP) are major regulators of extracellular matrix (ECM) synthesis and degradation. TIMP 1, a 28KDa protein, can inactivate stomelysin and collagenase activity (222). The mitogenic response of SSc fibroblasts to TIMP-1 has been reported as significantly greater than the response seen in normal fibroblasts, suggesting an important role for TIMP-1 as an autocrine growth factor in the fibrotic response in SSc (222).

The structural composition of the ECM is profoundly altered in affected SSc tissues. ECM components such as collagen type V regulate the organisation of ECM. Andrikopoulos *et al* demostrated that a homozygous deletion of the col5A2 gene in mice produced skin and eye abnormalitites, resembling the typical changes seen in human connective tissue disease. They concluded that type V collagen is a key

determinant in the assembly of tissue-specific matrices (223). In a fibrotic environment the balance between cells and the amount of surrounding ECM is disturbed and cellular interactions become altered. In comparison to normal fibroblasts scleroderma fibroblasts show enhanced adhesion to ECM components, possibly due to increased cellular receptors for ECM (224). Changes in the ECM environment of the fibroblast may contribute to the enhanced fibrotic response seen in SSc.

It is hypothesised that polymorphisms of genes involved in fibrosis may determine susceptibility to SSc. Gene polymorphisms may affect both the function and the level of protein production, and polymorphisms in genes involved in fibrosis could result in individuals with a predisposition to excessive fibrosis. Given the variation in the extent of skin fibrosis in different autoantibody subsets of disease, it is hypothesised that the genes involved in fibrosis may be associated with disease subtype. As functional polymorphisms for most of these candidate genes have not yet been mapped, investigation can only be done by association based analysis. Therefore, a strategy of using microsatellite markers that map either within the candidate gene or within a distance of one centimorgan (cM) of the gene has been used as described in chapter 2. The genes for six proteins involved in the fibrotic response (TGFB1, TGFB2, TGFB3, TIMP1, PDGFB and Col5A2) have been investigated in a case–control study using microsatellite markers.

A similar investigation, using the same group of patients, was carried out by Susol *et al.* in which, an association of microsatellite markers for TGFβ3, TGFβ2 and TIMP1 with SSc when compared to controls was identified (225). In this study patients were then stratified according to limited or diffuse disease. Analysis, by global chi-square with Monte-Carlo simulation, revealed a significant difference in allele frequency of the TGFβ2 marker in lcSSc patients compared to normal controls. The allele frequency distribution for the TGFβ3 marker was significantly different when dcSSc patients were compared to controls and lcSSc patients (110). In the present study patients are stratified according to autoantibody positivity. A comparison will be made with the data from Susol *et al.* (stratified according lcSSc/dcSSc) to investigate whether associations appear stronger or to be weakened with autoantibody stratification.

6.2: CHAPTER AIMS

The work in this chapter details an investigation of the polymorphisms of five candidate genes for proteins considered key in the SSc-fibrotic response, and their association with SSc and SSc autoantibody subgroups, by the use of microsatellite markers. The investigation was carried out by means of the following aims:

I. To screen a panel of microsatellite marker for candidate genes that encode proteins involved in fibrosis

II. To identify any microsatellite marker allele associations with SSc

III. To identify any microsatellite marker allele associations with specific autoantibody in SSc patients

6.3: MATERIALS AND METHODS

Patients and controls

One hundred and ninety-one, Caucasian, systemic sclerosis patients, from two centres were included in the investigation detailed in this chapter. Fifty-one individuals were patients of the Royal National Hospital for Rheumatic Diseases in Bath and the remaining 140 were enrolled from the Hope Hospital, Salford.

One hundred and ninety-six healthy individuals comprised the control group. Control samples were obtained from the Norfolk Family Health Service Authority Register, which comprises individuals who had registered with a general practitioner, and from blood donors in the Oxford area.

DNA extraction

DNA was extracted from EDTA treated blood samples as described in method 2.2.1.

Genotyping of microsatellite markers for candidate genes

The genes for five proteins involved in the fibrotic response (TGFβ1, TGFβ2, TGFβ3, PDGFB and COL5A2) were investigated by the use of microsatellite markers according to method 2.2.3. (Details of the microsatellite markers can be seen in table 2.1).

Autoantibody identification

Immunofluorescence, Ouchterlony double immunodiffusion and immunoprecipitation techniques were used to identify serological autoantibodies in all SSc patients, according to methods 2.4.2, 2.4.3 and 2.4.4 respectively.

Statistical analysis

Statistical analysis was carried out as previously described (section 2.5). All values have been corrected, (Bonferroni correction), to reduce the chance of false positive results due to multiple testing of the data.

6.4: RESULTS

6.4.1: Allele frequency analysis of microsatellite markers in SSc patients

For each microsatellite marker, associations with SSc were investigated by comparing allele frequencies of patients with those observed in the normal control population. Further analysis was carried out with stratification of patients according to the presence of specific autoantibodies. Observed allele frequencies for the microsatellite markers corresponding to the candidate genes investigated are shown in tables 6.1 to 6.7. The allele frequencies for all SSc cases, SSc cases stratified with regard to specific autoantibody and control subjects are detailed.

Microsatellite marker allele frequency comparisons between SSc patients and normal controls

The microsatellite marker d1S419, which maps within one cM of TGFβ2, was used to investigate association of this gene with SSc and TGFβ2. A significant increase in the frequency of allele 109 was observed in SSc patients compared to controls ($p=0.05$). D14S277, the microsatellite marker associated with the candidate gene TGFβ3 was significantly decreased in patients compared to controls ($p=0.05$). Allele 102 of the microsatellite marker that maps within PDGFB gene was raised in the SSc population when compared to normal controls, however this did not reach significance ($p=0.06$). No significant differences in allele frequencies for the microsatellite markers mapping for the other candidate genes investigated showed any significant differences in allele frequencies when compared to normal controls.

6.4.2: Allele frequency analysis of microsatellite markers in SSc patients stratified according to the presence of specific autoantibodies

Stratification according to the presence of specific autoantibodies

The microsatellite markers for the SSc candidate genes of interest have been investigated further to identify any association with specific autoantibody. Autoantibody specificities for centromere, topoisomerase-I, RNAP III, Jo-1, Pm-Scl, Ro, La and U1RNP proteins have been investigated. Anti-U3RNP autoantibody has not

been considered, as there were not a sufficient number of patients positive for this autoantibody for analysis (n=2).

Investigation of association of the microsatellite marker for PDGFB, (d22S284), with specific autoantibody

A significant increase in the frequency of allele 108 was observed in anti-Ro positive SSc patients when compared with normal controls (p=0.0065). In the ACA positive patients allele 108 was also raised in patients compared to controls, although this did not reach significance (p=0.065) (table 6.1).

Investigation of the association of the microsatellite marker for TGFβ2, (d1S419), with specific autoantibody

There were no significant differences in allele frequencies observed in any of the SSc autoantibody groups compared to normal controls. Allele 114 was raised in ATA positive patients and allele 115 was raised in anti-La patients, however these differences did not reach significance (p=0.09) (Table 6.2).

Investigation of the association of the microsatellite marker for COL5A2, (d2S389), with specific autoantibody

One significant difference in allele phenotype frequency was identified when marker d2S389, which maps to Col5A2 gene, was investigated. Allele 108 was significantly raised in ATA positive patients compared to controls (p=0.003) (Table 6.3).

Investigation of the association of microsatellite marker for PDGFB, (PDGFB), with specific autoantibody

The frequency of allele 102 was significantly raised in anti-Pm-Scl positive patients compared to controls (p=0.01) (Table 6.4).

Investigation of the association of microsatellite marker for TGFβ3, (d14S277) with specific autoantibody

Anti-La positive patients showed a significant increase in the frequency of allele 107 compared to controls (p=0.042). No other significant differences were observed in any of the other autoantibody groups. However, a slight decrease in allele 106 and increase

in allele 109 were identified in ARA III positive individuals compared to controls (p=0.07, without correction for the number of variables tested) (Table 6.5).

Investigation of the association of microsatellite marker for TGFβ1, (d19S400) with specific autoantibody

The frequency of allele 101 was significantly increased in anti-Ro positive patients compared to normal controls (p=0.001). No other alleles, in any of the other autoantibody groups showed any significant variation in frequency compared to normal controls for this marker (Table 6.6).

Investigation of the association of microsatellite marker for TIMP1, (dxS426) with specific autoantibody, in female patients

The microsatellite marker dxS426 was used to investigate association between SSc and TIMP1. This gene maps to the X chromosome and thus the data have been stratified according to gender. One hundred and sixty eight patients genotyped for this locus were female and 29 were male. Only the female patients have been considered here, as the number of men included in the study was too few (Table 6.7). No significant differences in allele frequencies of patients compared to controls were observed.

6.4.3: Comparison of previously reported associations of microsatellite marker alleles in patients stratified according to cutaneous involvement with patients stratified in the present study according to autoantibody status.

In the study by Susol *et al.*, which considered the same group of patients detailed in the present study, associations with microsatellite markers TGFβ3 and TIMP 1 with SSc patients were identified. It was hypothesised that if TGFβ3 and TIMP genes were involved in determining the degree of fibrosis, then a difference in the frequency of allele distribution between lcSSc and dcSSc patients would be expected.

When patients were analysed according to cutaneous involvement, a significant difference in the frequency of allele distribution for the microsatellite marker TGFβ3 was identified (p=0.03). Allele 106 was increased in lcSSc patients (lcSSc 18.7% *vs.* 8.8% dcSSc). Allele 109 was significantly increased in dcSSc patients (dcSSc 7.5% *vs.*

1.7% lcSSc). The results indicate that a gene polymorphism in linkage disequilibrium with TGFβ3 marker may have a role in determining the disease subtype.

Analysis of the microsatellite marker for TIMP 1 required stratification according to gender, as this gene maps to the sex chromosomes. Analysis did not show any significant differences in the distribution of allele frequency in female SSc patients. A significant difference in allele frequency distribution was identified between male SSc cases and controls (p=0.03). Allele 108 was found to be positively associated, and allele 114 negatively associated, with male SSc. It was concluded thus that a gene polymorphism within TIMP, which is in linkage disequilibrium with the marker DXS426 may exist that predisposes individuals to SSc. Stratification according to lcSSc and dcSSc did not show any significant differences in the distribution of allele frequency.

It is hypothesised that stratification according to autoantibody positivity may support gene associations and possibly define patient groups further. Tables 6.8 and 6.9 are direct comparisons of the data from Susol *et al*, stratified according to dcSSc or lcSSc with data from the present study. (Specific alleles of interest, identified from earlier analysis have been considered).

It can be seen from table 6.8 that there are similarities in the data from the two studies. ACA, as discussed earlier, is associated with lcSSc. The frequency of allele 106 is comparable in ACA positive and lcSSc patients (20.4% and 18.7%, respectively (controls 25.0%)), despite differences in the size of each population. A similar trend can be seen for allele 106 between ARA III positive patients and dcSSc patients (9.4% and 8.8%, respectively, (controls 25.0%)).

ARA III is often associated with dcSSc. The frequency of allele 109 for the microsatellite marker for TGFβ3 is raised in ARA III positive patients and dcSSc patients (9.4% and 7.5%, respectively) compared to controls. Conversely, the frequency of this allele in ACA positive patients and lcSSc patients is comparable to the control population (2.1% and 1.7%, respectively, compared to 2.3%).

The results from both studies are supportive of each other. They suggest that allele 106 for microsatellite marker for TGFβ is not associated with dcSSc or ARA III positive patients and allele 109 for this marker may render some individuals susceptible to the progressive form of SSc. The association with ARA III may also indicate an association of the allele with renal disease, a manifestation often associated with this autoantibody.

Table 6.9 shows a similar analysis of three alleles of interest for the microsatellite marker for TIMP 1. Significant allele frequency differences identified in the male SSc patients were lost on stratification for lc/dcSSc. Allele 108 was found to be positively associated, and allele 114 negatively associated, in the total male SSc group analysed by Susol *et al.*. Allele associations of this kind were not supported when patients were stratified according to autoantibody status.

Table 6.1: Allele frequencies for microsatellite marker d22S284

Allele	All Patients (n=191) (A=382) (%)	ACA (n=72) (A=144) (%)	ATA (n=20) (A=40) (%)	ARA III (n=16) (A=32) (%)	Anti-Jo-1 (n=6) (A=12) (%)	Pm-Scl (n=9) (A=18) (%)	Anti-Ro (n=9) (A=18) (%)	Anti-La (n=9) (A=18) (%)	Anti-U1RNP (n=8) (A=16) (%)	Normals (n=194) (A=388) (%)
101	4 (1.0)	3 (2.1)	0 (0.0)	1 (3.1)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	4 (1.0)
102	59 (15.4)	23 (16.0)	9 (22.5)	3 (9.4)	0 (0.0)	3 (16.7)	1 (5.6)	3 (16.7)	1 (6.3)	50 (13.0)
103	2 (0.5)	1 (0.7)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (0.5)
104	23 (6.0)	8 (5.6)	3 (7.5)	0 (0.0)	1 (8.3)	2 (11.1)	0 (0.0)	1 (5.6)	0 (0.0)	28 (7.2)
105	16 (4.2)	4 (2.8)	1 (2.5)	3 (9.4)	1 (8.3)	0 (0.0)	1 (5.6)	1 (5.6)	1 (6.3)	19 (5.0)
106	63 (16.4)	19 (13.2)	7 (17.5)	9 (28.1)	4 (33.3)	1 (5.6)	3 (16.7)	4 (22.2)	3 (18.8)	52 (13.4)
107	132 (34.6)	45 (31.3)	16 (40.0)	13 (40.6)	4 (33.3)	6 (33.3)	7 (38.9)	5 (27.8)	9 (56.3)	164 (42.3)
108	46 (12.0)	22 (15.3)	2 (5.0)	1 (3.1)	0 (0.0)	3 (16.7)	[1]6 (33.3)	3 (16.7)	2 (12.5)	28 (7.2)
109	27 (7.1)	14 (9.7)	2 (5.0)	1 (3.1)	1 (8.3)	3 (16.7)	0 (0.0)	1 (5.6)	0 (0.0)	29 (7.4)
110	4 (1.0)	2 (1.4)	0 (0.0)	0 (0.0)	1 (8.3)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	8 (2.1)
111	1 (0.3)	1 (0.7)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.3)
112	5 (1.3)	2 (1.4)	0 (0.0)	1 (3.1)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (0.5)
140	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.3)

Investigation of association between SSc and PDGFB, using microsatellite marker D22S284 which maps within 1cM of PDGFB. Allele 108 was significantly raised in anti-Ro positive SSc patients compared to normal controls: $p^1 = 0.0065$, with Bonferroni correction, OR=6.4(2.2-18.4).

Table 6.2: Allele frequencies for microsatellite marker d1S419

Allele	All Patients (n=189) (A=378) (%)	ACA (n=71) (A=142) (%)	ATA (n=20) (A=40) (%)	ARA III (n=15) (A=30) (%)	Anti-Jo-1 (n=6) (A=12) (%)	Pm-Scl (n=9) (A=18) (%)	Anti-Ro (n=9) (A=18) (%)	Anti-La (n=9) (A=18) (%)	Anti-U1RNP (n=8) (A=16) (%)	Normals (n=194) (A=388) (%)
102	9 (2.4)	5 (3.5)	0 (0.0)	0 (0.0)	0 (0.0)	1 (5.6)	0 (0.0)	0 (0.0)	2 (12.5)	13 (3.4)
103	1 (0.3)	0 (0.0)	1 (2.5)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (6.3)	1 (0.3)
104	10 (2.7)	6 (4.2)	0 (0.0)	1 (3.3)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	11 (2.8)
105	4 (1.1)	0 (0.0)	1 (2.5)	1 (3.3)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	4 (1.0)
106	54 (14.3)	15 (10.6)	5 (12.5)	2 (6.7)	1 (8.3)	7 (38.9)	3 (16.7)	6 (33.3)	3 (18.8)	58 (15.0)
107	32 (8.5)	16 (11.3)	3 (7.5)	3 (10.0)	0 (0.0)	3 (16.7)	2 (11.1)	1 (5.6)	1 (6.3)	20 (5.2)
108	18 (4.8)	6 (4.2)	1 (2.5)	0 (0.0)	0 (0.0)	1 (5.6)	1 (5.6)	1 (5.6)	1 (6.3)	21(5.4)
109	[1]11 (3.0)	3 (2.1)	0 (0.0)	2 (6.7)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (6.3)	1 (0.3)
110	26 (6.9)	8 (5.6)	3 (7.5)	4 (13.3)	2 (16.7)	0 (0.0)	4 (22.2)	3 (16.7)	2 (12.5)	31 (8.0)
111	104 (27.5)	50 (35.2)	10 (25.0)	6 (20.0)	5 (41.7)	3 (16.7)	1 (5.6)	1 (5.6)	5 (31.3)	129 (33.2)
112	34 (9.0)	14 (9.9)	3 (7.5)	4 (13.3)	0 (0.0)	1 (5.6)	3 (16.7)	2 (11.1)	0 (0.0)	50 (12.8)
113	33 (8.7)	11 (7.8)	6 (15.0)	3 (10.0)	1 (8.3)	2 (11.1)	2 (11.1)	1 (5.6)	0 (0.0)	24 (6.2)
114	19 (5.0)	4 (2.8)	5 (12.5)	2 (6.7)	1 (8.3)	0 (0.0.)	0 (0.0)	0 (0.0)	0 (0.0)	10 (2.6)
115	12 (3.1)	3 (2.1)	1 (2.5)	1 (3.3)	2 (16.7)	0 (0.0)	2 (11.1)	3 (16.7)	0 (0.0)	9 (2.3)
116	8 (2.1)	1 (0.7)	1 (2.5)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	4 (1.0)
118	1 (0.3)	0 (0.0)	0 (0.0)	1 (3.3)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.3)
119	2 (0.5)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
117	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.3)

Investigation of TGFβ2 by microsatellite marker D1S419, which maps within 1 cM of this candidate gene. A significant increase in the frequency of allele 109 was observed in SSc patients: [1]=0.05 with Bonferonni correction. OR=11.6(1.5-90.3).

Table 6.3: Allele frequencies for microsatellite marker d2S389

Allele	All Patients (n=188) (A=376) (%)	ACA (n=70) (A=140) (%)	ATA (n=20) (A=40) (%)	ARA III (n=16) (A=32) (%)	Anti-Jo-1 (n=6) (A=12) (%)	Pm-Scl (n=9) (A=18) (%)	Anti-Ro (n=9) (A=18) (%)	Anti-La (n=9) (A=18) (%)	Anti-U1RNP (n=8) (A=16) (%)	Normals (n=194) (A=388) (%)
101	2 (0.5)	0 (0.0)	0 (0.0)	0 (0.0)	1 (8.3)	0 (0.0)	0 (0)	0 (0.0)	0 (0.0)	4 (1.0)
103	26 (7.0)	11 (7.9)	5 (12.5)	2 (6.3)	0 (0.0)	0 (0.0)	3 (16.7)	3 (16.7)	2 (12.5)	26 (6.7)
105	6 (1.6)	3 (2.1)	0 (0.0)	1 (3.1)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (6.3)	7 (1.8)
106	68 (18.1)	19 (13.6)	7 (17.5)	5 (15.6)	3 (25.0)	5 (27.8)	5 (27.8)	6 (33.3)	1 (6.3)	69 (17.8)
107	121 (32.2)	48 (34.3)	13 (32.5)	10 (31.3)	2 (16.7)	7 (38.9)	5 (27.8)	4 (22.2)	5 (31.3)	106 (27.3)
108	5 (0.3)	0 (0.0)	[1]3 (7.5)	0 (0.0)	1 (8.3)	0 (0.0)	1 (5.6)	0 (0.0)	0 (0.0)	1 (0.3)
109	2 (0.5)	1 (0.7)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	3 (0.8)
110	4 (1.1)	1 (0.7)	1 (2.5)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (6.3)	0 (0)
111	27 (7.2)	11 (7.9)	2 (5.0)	3 (9.4)	2 (16.7)	0 (0.0)	1 (5.6)	2 (11.1)	0 (0.0)	28 (7.2)
112	37 (9.8)	15 (10.7)	4 (10.0)	2 (6.3)	0 (0.0)	1 (5.6)	1 (5.6)	1 (5.6)	4 (25.0)	38 (9.8)
113	14 (3.7)	10 (7.1)	0 (0.0)	2 (6.3)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	12 (3.1)
114	18 (4.8)	6 (4.3)	2 (5.0)	1 (3.1)	1 (8.3)	0 (0.0)	0 (0.0)	0 (0.0)	1 (6.3)	32 (8.2)
115	43 (11.4)	15 (10.7)	2 (5.0)	5 (15.6)	1 (8.3)	5 (27.8)	2 (11.1)	2 (11.1)	1 (6.3)	55 (14.2)
116	4 (1.1)	0 (0.0)	1 (2.5)	1 (3.1)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	7 (1.8)
119	1 (0.3)	0 (0.0)	0 (0.0)	0 (0.0)	1 (8.3)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)

Investigation of candidate gene Col5A2 by the use of microsatellite marker D2S389 which maps wihin 1cM of the gene. A significant increase in the frequency of allele 108 was observed in ATA positive patients when compared to controls: [1]=0.003 with Bonnferoni correction, OR=31.7(3.1-309).

Table 6.4: Allele frequencies for microsatellite marker pdgfb

Allele	All Patients (n=195) (A=390) (%)	ACA (n=68) (A=136) (%)	ATA (n=20) (A=40) (%)	ARA III (n=16) (A=32) (%)	Anti-Jo-1 (n=6) (A=12) (%)	Anti-Pm-Scl (n=8) (A=16) (%)	Anti-Ro (n=9) (A=18) (%)	Anti-La (n=9) (A=18) (%)	Anti-U1RNP (n=8) (A=16) (%)	Normals (n=195) (A=390) (%)
101	239 (61.3)	93 (68.4)	21 (52.5)	21 (65.6)	7 (58.3)	10 (62.5)	17 (94.4)	13 (72.2)	13 (81.3)	254 (65.1)
102	24 (6.2)	8 (5.9)	2 (5.0)	2 (6.3)	0 (0.0)	[1]3 (18.8)	0 (0.0)	0 (0.0)	0 (0.0)	9 (2.3)
103	12 (3.1)	4 (2.9)	3 (7.5)	1 (3.1)	1 (8.3)	0 (0.0)	1 (5.6)	1 (5.6)	0 (0.0)	13 (3.3)
105	3 (0.8)	1 (0.7)	0 (0.0)	2 (6.3)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	3 (0.8)
106	9 (2.3)	2 (1.5)	4 (10.0)	1 (3.1)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (12.5)	12 (3.1)
107	65 (16.7)	19 (14.0)	7 (17.5)	1 (3.1)	2 (16.7)	1 (6.3)	0 (0.0)	4 (22.2)	1 (6.3)	68 (17.4)
108	28 (7.2)	9 (6.6)	2 (5.0)	4 (12.5)	2 (16.7)	2 (12.5)	0 (0.0)	0 (0.0)	0 (0.0)	30 (7.7)
109	2 (0.5)	0 (0.0)	1 (2.5)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.3)
120	1 (0.5)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0)

Investigation of PDGFB using the microsatellite marker pdgfb, which maps with the PDGFB gene. The frequency of allele 102 was significantly increased in Pm-Scl: [1]=0.01 with Bonfferoni correction, OR=9.8(2.4-40.4).

Table 6.5: Allele frequencies for microsatellite marker d14S277.

Allele	All Patients (n=189) (A=378) (%)	ACA (n=71) (A=142) (%)	ATA (n=20) (A=40) (%)	ARA III (n=16) (A=32) (%)	Anti-Jo-1 (n=5) (A=10) (%)	Pm-Scl (n=9) (A=18) (%)	Anti-Ro (n=9) (A=18) (%)	Anti-La (n=9) (A=18) (%)	Anti-U1RNP (n=8) (A=16) (%)	Normals (n=195) (A=390) (%)
102	23 (6.1)	11 (7.7)	3 (7.5)	1 (3.1)	1 (10.0)	1 (5.6)	0 (0.0)	0 (0.0)	1 (6.3)	19 (4.9)
103	19 (5.0)	8 (5.6)	3 (7.5)	1 (3.1)	0 (0.0)	1 (5.5)	1 (5.6)	0 (0.0)	0 (0.0)	23 (5.9)
104	5 (1.3)	1 (0.7)	0 (0.0)	2 (6.3)	0 (0.0)	1 (5.6)	1 (5.6)	0 (0.0)	0 (0.0)	4 (1.0)
105	19 (5.0)	4 (2.8)	1 (5.0)	4 (12.5)	1 (10.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (6.3)	10 (2.6)
106	[1]63 (16.7)	29 (20.4)	3 (7.5)	3 (9.4)	1 (10.0)	5 (27.8)	1 (5.6)	1 (5.6)	3 (18.8)	98 (25.1)
107	165 (43.7)	60 (42.3)	17 (42.5)	13 (40.6)	5 (50.0)	5 (27.8)	10 (55.6)	[2]14 (77.8)	8 (50.0)	158 (40.4)
108	62 (16.4)	23 (16.2)	8 (20.0)	4 (12.5)	2 (20.0)	3 (16.7)	4 (22.2)	2 (11.1)	2 (12.5)	54 (13.8)
109	12 (3.2)	3 (2.1)	3 (7.5)	3 (9.4)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (6.3)	9 (2.3)
110	3 (0.8)	1 (0.7)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	9 (2.3)
111	1 (0.3)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0)
114	1 (0.3)	1 (0.7)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (0.5)
115	1 (0.3)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (5.6)	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.3)
117	1 (0.3)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (5.6)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0)
120	3 (0.8)	0 (0.0)	1 (5.0)	1 (3.1)	0 (0.0)	0 (0.0)	1 (5.6)	1 (5.6)	0 (0.0)	1 (0.3)

Investigation of TGFβ3 by the use of microsatellite marker D14S277 which maps within 1cM of the candidate gene. A significant decrease in allele frequency was observed in allele 106 in the SSc patient group and a significant increase in allele 107 in anti-La positive patients: p^1=0.05 (OR=0.6(0.4-0.8), p^2=0.04 (OR=5.1(1.7-16.0) respectively, with Bonferroni correction.

Table 6.6: Allele frequencies for microsatellite marker d19S400

Allele	All Patients (n=188) (A=376) (%)	ACA (n=72) (A=144) (%)	ATA (n=20) (A=40) (%)	ARA III (n=16) (A=32) (%)	Anti-Jo-1 (n=6) (A=12) (%)	Pm-Scl (n=9) (A=18) (%)	Anti-Ro (n=9) (A=18) (%)	Anti-La (n=9) (A=18) (%)	Anti-U1RNP (n=8) (A=16) (%)	Normals (n=191) (A=382) (%)
101	1 (0.3)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	[1]2 (11.1)	0 (0.0)	0 (0.0)	1 (0.3)
102	45 (12.0)	21 (14.6)	2 (5.0)	3 (9.4)	3 (25.0)	3 (16.7)	0 (0.0)	1 (5.6)	3 (18.8)	39 (10.2)
103	2 (0.5)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
104	13 (3.5)	6 (4.2)	1 (2.5)	3 (9.4)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (6.3)	11 (2.9)
105	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (0.5)
106	16 (4.3)	6 (4.2)	1 (2.5)	1 (3.1)	0 (0.0)	1 (5.6)	1 (5.6)	1 (5.6)	0 (0.0)	20 (5.2)
108	99 (26.3)	30 (20.8)	14 (35.0)	8 (25.0)	4 (33.3)	4 (22.2)	7 (38.9)	7 (38.9)	3 (18.8)	101 (26.3)
110	56 (14.9)	24 (14.9)	8 (20.0)	5 (15.6)	2 (16.7)	4 (22.2)	2 (11.1)	2 (11.1)	1 (6.3)	48 (12.5)
111	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.3)
112	46 (12.2)	17 (10.6)	5 (12.5)	5 (15.6)	2 (16.7)	0 (0.0)	1 (5.6)	2 (11.1)	5 (31.3)	60 (15.6)
114	62 (16.5)	23 (14.3)	6 (15.0)	5 (15.6)	0 (0.0)	3 (16.7)	4 (22.2)	4 (22.2)	2 (12.5)	65 (16.9)
115	1 (0.3)	1 (0.6)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
116	29 (7.7)	11 (6.8)	1 (2.5)	1 (3.1)	1 (8.3)	2 (11.1)	0 (0.0)	1 (5.6)	1 (6.3)	28 (7.3)
118	5 (1.3)	0 (0.0)	0 (0.0)	1 (3.1)	0 (0.0)	1 (5.6)	1 (5.6)	0 (0.0)	0 (0.0)	6 (1.5)
120	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (0.5)

Investigation of association of TGFβ1 with SSc by the use of microsatellite marker d19S400. A significant increase in the frequency of allele 101 was observed in anti-Ro positive patients when compared to controls: [1]=0.0015 with Bonferroni correction, OR=47.6(4.1-553.0).

Table 6.7: Allele frequencies for microsatellite marker dXS426 in females

Allele	All Females (n=168) (A=336) (%)	ACA (n=67) (A=134) (%)	ATA (n=13) (A=26) (%)	ARA III (n=13) (A=26) (%)	Anti-Jo-1 (n=5) (A=10) (%)	Pm-Scl (n=8) (A=16) (%)	Anti-Ro (n=6) (A=12) (%)	Anti-La (n=7) (A=14) (%)	Anti-U1RNP (n=6) (A=12) (%)	Normals (n=100) (A=200) (%)
103	1 (0.3)	1 (0.7)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
104	22 (6.5)	11 (8.2)	3 (11.5)	2 (7.7)	0 (0.0)	0 (0.0)	1 (8.3)	1 (7.1)	0 (0.0)	23 (11.5)
105	164 (48.8)	69 (51.5)	12 (46.2)	16 (61.5)	7 (70.0)	11 (68.8)	6 (50.0)	8 (57.1)	7 (58.3)	109 (54.5)
106	33 (9.8)	[1]13 (9.7)	3 (11.5)	0 (0.0)	1 (10.0)	3 (18.8)	2 (16.7)	1 (7.1)	1 (8.3)	15 (7.5)
107	2 (0.6)	0 (0.0)	2 (7.7)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
108	3 (0.9)	2 (1.5)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.5)
109	3 (0.9)	2 (1.5)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
110	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.5)
111	2 (0.6)	1 (0.7)	1 (3.9)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
113	3 (0.9)	0 (0.0)	0 (0.0)	1 (3.8)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.5)
114	49 (14.6)	21 (15.7)	3 (11.5)	2 (7.7)	1 (10.0)	2 (12.5)	3 (25.0)	3 (21.4)	2 (16.7)	35 (17.5)
115	15 (4.5)	6 (4.5)	1 (3.9)	2 (7.7)	0 (0.0)	0 (0.0)	0 (0.0)	1 (7.1)	0 (0.0)	4 (2.0)
116	8 (2.4)	2 (1.5)	1 (3.9)	2 (7.7)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (16.7)	5 (2.5)
117	1 (0.3)	0 (0.0)	0 (0.0)	0 (0.0)	1 (10.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (1.0)
118	1 (0.3)	0 (0.0)	0 (0.0)	1 (3.8)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.5)
119	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.5)
132	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (1.0)

Investigation of association between TIMP 1 and SSc by use of the microsatellite marker DXS426, which maps within 1cM of this candidate gene. TIMP 1 maps to the X chromosome, thus patients and controls have been stratified according to gender and only data from females is shown. No significant differences in the allele frequencies of patients compared to controls were identified.

Table 6.8: A direct comparison of the frequencies of two alleles of the microsatellite marker for TGFβ3; between patients stratified according to cutaneous classification of disease, or, ACA or ARA III positivity.

Allele	Data from Susol *et al.*		Data from present study		Controls
	lcSSc	dcSSc	ACA	ARA III	
	[n=150] [A=300] (%)	[n=40] [A=300] (%)	[n=71] [A=142] (%)	[n=16] [A=390] (%)	[n=195] [A=390] (%)
106	56 (18.7)	7 (8.8)	29 (20.4)	3 (9.4)	98 (25.0)
109	5 (1.7)	6 (7.5)	3 (2.1)	3 (9.4)	9 (2.3)

The frequencies of the alleles shown are comparative between ACA positive patients and lcSSc patients, and ARA III and dcSSc SSc patients. This supports associations of autoantibody with disease subtype, and the association of specific polymorphisms with disease subgroups.

Table 6.9: A direct comparison of the frequencies of three alleles of the microsatellite marker for TIMP 1; between male and female patients stratified according to cutaneous classification of disease and those who were ACA or ARA III positive

	Allele	Data from Susol *et al.*		Data from present study		Controls
		lcSSc	dcSSc	ACA	ARA III	
Male		[n=16]	[n=11]	[n=5]	[n=2]	[n=51]
		[A=32]	[A=22]	[A=10]	[A=4]	[A=102]
		(%)	(%)	(%)	(%)	(%)
	108	3 (9.4)	2 (9.1)	1 (10.0)	0 (0.0)	2 (2.0)
	114	3 (9.4)	0 (0.0)	0 (0.0)	0 (0.0)	24 (23.8)
	115	1 (3.1)	0 (0.0)	1 (10.0)	0 (0.0)	6 (5.9)
Female		[n=127]	[n=29]	[n=67]	[n=13]	[n=100]
		[A=254]	[A=58]	[A=134]	[A=26]	[A=200]
		(%)	(%)	(%)	(%)	(%)
	108	3 (1.2)	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.5)
	114	47 (18.5)	2 (3.4)	21 (15.7)	2 (7.7)	35 (17.5)
	115	4 (1.6)	5 (8.6)	6 (4.5)	2 (7.7)	4 (2.0)

Significant differences in distribution of allele frequency were lost on stratification of patients according to limited or diffuse disease. No significant differences in allele frequency distribution were identified when patients were stratified according to autoantibody positivity.

6.5: DISCUSSION

In this study a range of microsatellite markers have been used to investigate candidate genes for SSc. The markers map very close to (<1 cM), (or within), the gene of interest and it is anticipated that strong linkage disequilibrium will exist between markers and genes, thus association with a microsatellite marker is likely to reflect association with a disease polymorphism within the gene sequence. Gene polymorphisms may affect protein production and function, thus specific polymorphisms of genes involved in fibrosis may leave an individual susceptible to excessive fibrosis. Awad *et al.* demonstrated a variation in TGFβ1 production with genetic polymorphism and identified a specific allele associated with increased TGFβ1 production and pre-transplant fibrotic pathology (226).

The effects of TGFβ are widespread. Although all three isoforms of TGFβ can induce ECM production, studies have suggested that each isoform may not equally contribute to this process. It has been proposed that TGFβ1 and TGFβ2 may have a more central role in excess ECM production, causing fibrosis and scar formation, whilst TGFβ3 may be more involved in the subsequent down-regulation of ECM matrix production, possibly by down-regulating TGFβ1 production (227;228). Thus it has been suggested that the high levels of TGFβ1, TGFβ2 and TGFβ3 in SSc skin may indicate that either insufficient TGFβ3 is being produced or that TGFβ3 is not functionally able to down-regulate the production/action of TGFβ1 (110). It is suggested that if the levels or functionality of TGFβ2 and TGFβ3 are altered, due to a particular gene polymorphism, leading to excess TGFβ2 and reduced TGFbβ3 activity, an individual may be predisposed to fibrosis (110). The ratio of TGFβ isoforms, particularly that of TGFβ1 relative to TGFβ3, has been suggested to be a significant factor in reducing scarring and fibrosis (227), thus it may be that a particular *combination* of genetic polymorphisms may create an environment that makes individuals susceptible to fibrosis.

A difference in distribution of TGFβ3 microsatellite marker alleles between lcSSc and dcSSc patients was identified in the study by Susol *et al.* indicating a possible role for TGFβ3 in determining the degree of cutaneous fibrosis the patient develops (110). In the present study a significant increase in the allele 107, for this marker, was identified

in anti-La positive SSc patients compared to controls. Susol *et al.* identified a significant increase in the frequency of allele 106 in lcSSc patients compared to dcSSc patients, and a significant decrease in the frequency of allele 109 in lcSSc patients compared to dcSSc patients, for the microsatellite marker for TGFβ3. Interestingly an increase in frequency of allele 109, and a decrease in allele 106, was observed in ARA III positive patients in this study (although after correction the values no longer reached significance). Patients with ARA III have the greatest risk of dcSSc (45), thus an increase of allele 109 in ARA III positive patients supports the observation by Susol *et al.* of this allele being raised in dcSSc patients. The significant increase of allele 106 in lcSSc patients reported by Susol *et al.* is also supported by the observation of a decreased frequency of this allele in patients with ARA III in the present study. Defining patients by autoantibody may allow those at risk of developing diffuse cutaneous disease to be identified more easily.

The marker for TGFβ2 had a significantly different allele frequency distribution in lcSSc patients compared with controls in the study by Susol *et al.*. An association with this allele was also identified in the present study when SSc patients were compared to controls, but not when patients were stratified according to autoantibody. These results indicate that the marker for TGFβ2 may not be associated with clinical or autoantibody subset of disease.

Significant differences in allele frequencies were observed in anti-Ro and anti-Pm-Scl positive patients for the markers for PDGFB: D22S284 and pdgfb respectively, in ATA patients for Col5A2 and in anti-Ro positive patients for TGFβ1. However the numbers of individuals in each of these groups were very small and thus their true significance remains doubtful, particularly in the absence of any comparative published data.

This study has identified associations of microsatellite markers for TGFβ2 and TGFβ3, which are generally supported by the one published report considering this area (110). The results indicate that polymorphisms within TGFβ2, TGFβ3 may exist that

predispose individuals to SSc. Increased numbers of patients positive for specific autoantibodies will allow a more meaningful analysis to be carried out in the future.

CHAPTER 7

DISCUSSION

7: DISCUSSION

The aim of the work detailed in this thesis was to further understanding of the aetio-pathogenesis of SSc by; investigating the significance, and pattern, of autoantibody production, identifying possible genetic predetermination or restriction of autoantibody generation and exploring the relationship of these two factors with disease profile, course and outcome.

The pathogenesis of SSc is unknown. However, the presence of particular autoantibodies, which are often indicative of clinical expression, disease course, and overall severity, are unlikely to play a directly pathogenic role, but are certainly reliable markers of, and an invaluable aid to, the diagnosis and prognosis of individual patients. The mechanism underlying autoantibody production and the pattern of autoantibodies seen in SSc, the occurrence of distinct clinical subgroups, and the apparent associations between the two, may be held in an underlying genetic predeterminant. It is this concept that has formed the basis of this research.

This study began with an investigation and comparison of the HLA background of a group of Caucasian SSc patients, who had been positively identified for either of the major serological SSc autoantibodies: ATA or ARA III. ATA, ARA III and ACA (the latter autoantibody was considered separately) are regarded as mutually exclusive, a phenomenon which has been supported in this study. It was hypothesised that HLA associations would become stronger when autoantibody subgroups were considered. Results from this study confirm the previously reported association of HLA DR5 with ATA. More specifically this association was identified with the HLA DRB1*1101-1104 alleles, and not HLA DRB1*1201-1202, which also comprises DR5. An association of HLA DQB1*0301 with ATA was confirmed and an association with HLA-DQB1*0602 was identified. Within the ARA III autoantibody group no significant HLA associations were found. Fanning *et al.* also failed to identify any HLA associations in ARA III positive SSc patients. Previously reported associations of specific amino acid loci (HLA DQB1: Y: 30, DQB1: ^{71}TRAELDT^{77} and DQB1: Y: 26) with ATA were also supported. Again such associations were not present in the ARA III positive SSc population.

T cell proliferative responses of ATA positive SSc patients and normal individuals, to topoisomerase I supported those observations reported by Kuwana *et al.* (85), and also the association of HLA DR11 in these individuals. This suggests that HLA DR11 related alleles might facilitate a T cell driven ATA response. When a genetic model, regarding the presence of HLA DQB1: Y: 30 homozygosity in individuals able to mount a T cell proliferative responses to topoisomerase I, and an absence or heterozygosity for HLA DR4 in those who did not, was applied to the data from this study and from Kuwana *et als* study, it could account for 88% of patients.

In conclusion, work from this part of the study has supported previous HLA DR and DQ allele and amino acid loci associations in patients with ATA. These associations are not apparent in ARA III positive patients and, thus, it may be concluded that these two groups of patients, defined by mutually exclusive autoantibodies, are genetically distinct. However, mutual exclusivity of these autoantibody groups could not be explained by the genetic analysis carried out. That is not to say that a genetic component is not responsible.

Overall, this part of the investigation has identified HLA associations in patients with regard to their autoantibody status. These autoantibody associations become stronger when particular amino acid loci are considered. The consideration of other loci and possibly a combination of loci may strengthen or define associations further. The proliferative response of T cells from normal individuals provides evidence that; autoreactive T cells are part of the normal immune system repertoire, the response is genetically restricted, and also suggests that there is an unidentified factor initiating disease.

As discussed in the main introduction to this thesis in order for tolerance to be broken at the level of T cells, a general requirement is the presentation of cryptic epitopes of an autoantigen. A requirement for this is the provision of a self antigen in an altered context, as described by Rosen *et al.* (44). Topoisomerase I appears to be susceptible to cleavage by reactive oxygen species in a metal cation-dependent manner (43). Such modification of this protein, which may perhaps be driven by the generation of reactive oxygen species as a result of ischaemia reperfusion injury, may result in uniquely

expressed cryptic epitopes becoming exposed to the immune system initiating a T cell driven immune response.

The second part of the study considered ethnic variation in the autoimmune response and clinical expression of SSc. It was hypothesised that if HLA is a determinant of autoantibody profile, and is associated with disease course and/or outcome, then associations would be expected to remain the same independent of race. Of particular consideration was whether different HLA frequencies existed in the different ethnic groups, and if they did then could these differences explain the variance in autoantibody incidence and disease subtype in alternative races. Differences in SSc/HLA associations in alternative ethnic populations have previously been acknowledged. In the present study overall autoantibody profiles were indeed different between the races and supported previous reports. HLA frequencies were also different in each population. When HLA associations with autoantibody were investigated, associations were identified, and were in accordance with the literature, regardless of racial group. Thus overall the difference in the frequency of HLA alleles, serological autoantibody and clinical profile were relative within each racial group, and were quite different between the two populations. The proposed relationship between these three factors is thus supported, as is the hypothesis that HLA plays a significant role in SSc. This study provides evidence that HLA background, autoantibody profile and clinical course are ethnically variable however associations between these three factors are independent of ethnicity. Further, autoantibody mutual exclusivity, which was observed in both races, implies that specific processes give rise to a particular autoimmune response that may be, at least in part, genetically restricted. Overall, results from this part of the study indicate that the autoimmune response is predetermined – partially genetically and by other predisposing factors such as environmental exposure.

This study then went on to consider the autoimmune response directed against the centromere proteins. ACA form the third major autoantibody group in SSc, however ACA are not specific to SSc. To characterise the autoimmune response to ACA this part of the study considered all ACA positive patients identified through a rheumatology clinic. In concordance with previous reports ACA was identified in a spectrum of rheumatological conditions, where the majority of patients had lcSSc. The dcSSc variant was not associated with ACA, and in this particular population was not

encountered, which also supports current literature. Results from this study found that more than 91% of ACA patients, identified through a rheumatological setting, had lcSSc and/or a form of arthritis. ACA is generally regarded as a marker for a relatively benign disease course, though there is evidence from this study to suggest that for a minority of patients this is not the case. Previous associations of ACA with HLA DR1 and DR4 have been supported and an association with HLA DQB1*0501 has also been identified. In fact HLA DRB1*0101-0104 and/ or HLA DRB1*0401-0411 was present in 75% of patients suggesting that possession of at least one of these alleles predisposes individuals to, but is not an absolute requirement for, the ACA immune response, and further that HLA DR1 and/ or DR4 confers susceptibility in some individuals to lcSSc and/or RA in particular. Previous associations of amino acids at specific HLA DQB1 loci could not be supported in this study. It would be interesting to consider amino acid loci when patients are stratified according to their reactivity toward the CENP antigens.

Evidence has been provided in this study for an association between polymorphisms of genes involved in fibrosis, identified through linkage disequilibrium with a polymorphism of the microsatellite marker for the gene of interest. Such associations have been identified with markers for TGFβ3 and TIMP 1. This implies that polymorphisms within these genes may exist that predispose individuals to SSc. An association with TGFβ3 was supported by the results from the comparative study by Susol *et al.* where the same group of patients were considered, but stratified according to disease subtype. Results from the present study support the hypothesis that a polymorphism of TGFβ3, may have a role in determining whether a patient will have limited or diffuse disease. Identifying the association of this polymorphism within the ARA III autoantibody group may allow a more precise sub-grouping of patients; thus not only allowing determination of the degree of skin involvement but may also indicate the risk of internal manifestations and overall disease course. Thus stratification according to autoantibody status can support associations observed when patients are subgrouped according to disease subtype and may offer a potentially more sensitive tool for diagnosis and prognosis.

The present study has identified genetic characteristics, which distinguish autoantibody groups identified in SSc. Differences have been identified between the ACA autoantibody response, and genetic and clinical characteristics of ATA SSc patients, and

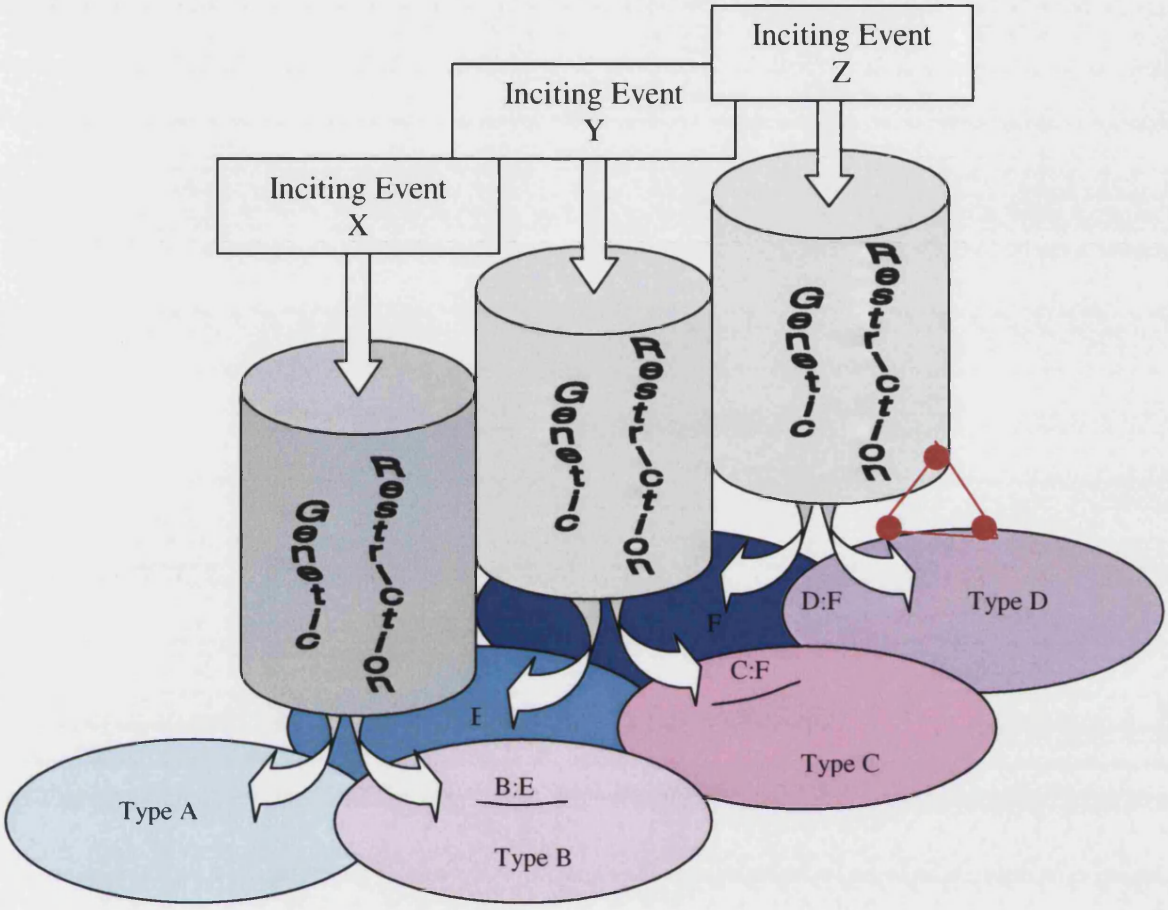
normal individuals who posses topoisomerase I specific autoreactive T cells. Autoantibody and HLA associations are independent of ethnicity; where autoantibody frequency alters in each race, according to HLA background.

To define associations of this nature further, more sensitive HLA techniques, which are now available, need to be employed. Since the work for this thesis began there have been substantial developments in genetic technology. Speed, automation and high throughput were not phrases associated with the PCR work carried out at the beginning of this project. With the availability of sophisticated genetic technologies there is great potential for extensive analysis of the gentetic contribution to SSc. An initial next step for this work would be to carry out HLA DPB1 typing. Associations with alleles at this locus have recently been emerging with SSc, and further, with SSc autoantibody groups (229). Any future work should also undertake the identification of additional candidate genes in SSc. Gene expression analysis by subtractive hybridisation techniques could be used to identify genes that are specifically expressed in disease tissue compared to normal tissue. Comparisons of the expression levels of genes, in different samples could be made, using differential display analyses. cDNA microarray technology allows simultaneous monitoring of differential expression of many genes at one time. Comparisons of gene expression profiles of healthy controls compared to SSc patients may reveal genes associated with SSc. Complex disease gene localisation has accelerated with single nucleotide polymorphism (SNP) mapping. Identification of SNPs occurring within the MHC could further elucidate genetic contribution to the disease. Identification of susceptibility genes will enhance understanding of the mechanisms of the disease process culminating SSc.

Only when clinical, genetic typing and autoantibody specificities are considered in their smallest components will any precise associations existing between them become defined. However, it is acknowledged that what these subgroups are exactly is not known. Larger patient numbers would increase the statistical power of the analysis carried out here. When more intricate autoantibody patterns are considered however, with the rarity of SSc, this is indeed a problem. Nevertheless: existing relationships of HLA-autoantibody-clinical profile have been supported, further associations defined and new ones identified and proposed.

It is possible that 'HLA and Autoantibody' association and 'clinical profile' are independent consequences of an inciting event such as an environmental trigger of some kind, (which, may differ in different cases of SSc). Fanning *et al.* proposed two possibilities regarding the pathogenesis of SSc and trying to explain the mutual exclusivity of autoantibodies observed in this disease. Firstly it was suggested that the three mutually exclusive autoantibody groups represent three distinct disease entities, and second the possibility that these groups have a common origin, which is modified by HLA specificity into the serologically defined disease subgroups. Their extensive HLA-autoantibody analysis failed to show associations that were strong enough to be wholly responsible for the observed mutual exclusivity. Therefore the separate disease hypothesis was supported. Along these lines one may consider that in fact both theories are important as is represented in figure 7.1.

Fig 7:1 Representation of a hypothesis for the pathogenesis of scleroderma



A hypothesis for the mechanism of disease pathogenesis in SSc, by which distinct disease subgroups are given rise to, where by: Clinical and serological expression of disease is dictated primarily by the inciting event and then by genetic restriction; potentially giving rise to a number of disease variants. Disease variants may be distinct or overlap with those derived from an alternative inciting event and genetic background (e.g. A or B, or B:E respectively). Thus variants may be identified by clinical and serological expression and genetic background, as described in figure 1.3, and represented by the red triangle. The red triangle is representative of the triad hypothesis proposed in chapter 1 (fig. 1.3). Consequently numerous disease variants may exist.

APPENDICES

APPENDIX I

Primers used in the identification of HLA-DRB1 and HLA-DQB1 typing by PCR-SSP

Primers for amplification of HLA-DQB1 alleles

Primer Mix	5'-primer	$^{5'}$sequence$^{3'}$	3'-primer	$^{5'}$sequence$^{3'}$	Amplified specificities
1	B-5'01	CGGAGCGCGTGCGGGG	B-3'01	GCTGTTCCAGTACTCGGCAA	DQB1*0501
2	B-5'02	TGCGGGGTGTGACCAGAC	B-3'02	TGTTCCAGTACTCGGCGCT	DQB1*0502
3	B-5'02	TGCGGGGTGTGACCAGAC	B-3'03	GCGGCGTCACCGCCCGA	DQB1*0503
4	B-5'03	GCCATGTGCTACTTCACCAAT	B-3'04	CACCGTGTCCAACTCCGCT	DQB1*0601
5	B-5'04	CGTGCGTCTTGTGACCAGAT	B-3'05	GCTGTTCCAGTACTCGGCAT	DQB1*0602
6	B-5'05	GGAGCGCGTGCGTCTTGTA	B-3'05	GCTGTTCCAGTACTCGGCAT	DQB1*0603, DQB1*0608
7	B-5'06	CGTGTACCAGTTTAAGGGCA	B-3'06	GCAGGATCCCGCGGTACC	DQB1*0604
8	B-5'07	GTGCGTCTTGTGAGCAGAAG	B-3'07	GCAAGGTCGTGCGGAGGCT	DQB1*0201/0202
9	B-5'08	GACGGAGCGCGTGCGTCT	B-3'08	CTGTTCCAGTACTCGGCGG	DQB1*0201, DQB1*0302
10	B-5'09	GACGGAGCGCGTGCGTTA	B-3'09	AGTACTCGGCGTCAGGCG	DQB1*0301, DQB1*0304
11	B-5'08	GACGGAGCGCGTGCGTTA	B-3'09	AGTACTCGGCGTCAGGCG	DQB1*0302, DQB1*0303
12	B-5'08	GACGGAGCGCGTGCGTTA	B-3'10	CTGTTCCAGTACTCGGCGT	DQB1*0303
13	B-5'10	CACCAACGGGACCGAGCT	B-3'11	GGTAGTTGTGTCTGCATACG	DQB1*0401
14	B-5'11	CACCAACGGGACCGAGCG	B-3'11	GGTAGTTGTGTCTGCATACG	DQB1*0402

APPENDIX II

Primers for amplification of HLA-DRB1 alleles

Primer Mix	5'-primer	5'sequence3'	3'-primer	5'sequence3'	Amplified specificities
1.1	5,01	TTGTGGCAGCTTAAGTTTGAAT	3'15	CCGCCTCTGCTCCAGGAG	DRB1*0101, DRB1*0102
1.2	5'01	TTGTGGCAGCTTAAGTTTGAAT	3'10	CCCGCTCGTCTTCCAGGAT	DRB1*0103
15	5'02	TCCTGTGGCAGCCTAAGAG	3'01	CCGCGCCTGCTCCAGGAT	DRB1*1501-1502
16	5'02	TCCTGTGGCAGCCTAAGAG	3'02	AGGTGTCCACCGCGGCG	DRB1*1601-1602
03	5'03	TACTTCCATAACCAGGAGGAGA	3'03	TGCAGTAGTTGTCCACCCG	DRB1*0301-0302
17	5'06	GACGGAGCGGGTGCGGTA	3'048	CTGCACTGTGAAGCTCTCCA	DRB1*0301
18	5'03	TACTTCCATAACCAGGAGGAGA	3'047	CTGCACTGTGAAGCTCTCAC	DRB1*0302,1302, 1305, 1402, 1403
4	5'04	GTTTCTTGGAGCAGGTTAAACA	3'047	CTGCACTGTGAAGCTCTCAC	DRB1*0401-0411
			3'048	CTGCACTGTGAAGCTCTCCA	
7	5'07	CCTGTGGCAGGGTAAGTATA	3,079	CCCGTAGTTGTGTCTGCACAC	DRB1*0701-0702
8mod	5'08	AGTACTCTACGGGTGAGTGTT	3'045	TGTTCCAGTACTCGGCGCT	DRB1*0801-0805
			3'18	GCTGTTCCAGTACTCGGCAT	
09	5'09	GTTTCTTGAAGCAGGATAAGTTT	3'079	CCCGTAGTTGTGTCTGCACAC	DRB1*0901
10	5'10	CGGTTGCTGGAAAGACGCG	3'047	CTGCACTGTGAAGCTCTCAC	DRB1*1001
11	5'05	GTTTCTTGGAGTACTCTACGTC	3'06	CTGGCTGTTCCAGTACTCCT	DRB1*1101-1104
12	5'08	AGTACTCTACGGGTGAGTGTT	3'08	CACTGTGAAGCTCTCCACAG	DRB1*1201-1202
13.1	5'03	TACTTCCATAACCAGGAGGAGA	3'10	CCCGCTCGTCTTCCAGGAT	DRB1*1301-1302
13.2	5'05	GTTTCTTGGAGTACTCTACGTC	3'045	TGTTCCAGTACTCGGCGCT	DRB1*1303-1304
13.3	5'03	TACTTCCATAACCAGGAGGAGA	3'17	CCCGCCTGTCTTCCAGGAA	DRB1*1305
14.1	5'05	GTTTCTTGGAGTACTCTACGTC	3'11	TCTGCAATAGGTGTCCACCT	DRB1*1401,1404,1405
	+5'08	AGTACTCTACGGGTGAGTGTT			
14.2	5'03	TACTTCCATAACCAGGAGGAGA	3'12	TCCACCGCGGCCCGCC	DRB1*1305,1402,1403
14.3	5'04	GTTTCTTGGAGCAGGTTAAACA	3'19	CTGTTCCAGTGCTCCGCAG	DRB1*1410

APPENDIX III

10% acrylamide gel

For 1 lower gel.

Double distilled, filter purified water	12.4ml
Lower gel buffer (1.5M Tris/HCl pH8.8, 0.4%w/v SDS)	7.5ml
Acrylamide (30% (w/v) acrylamide 937.5:1) bisacrylamide)	10.0ml
10% aqueous ammonium persulphate	150.0ul
TEMED	22.5µl

5% acrylamide gel

For 1 upper gel

Double distilled, filter purified water	5.9ml
Upper gel buffer (0.5M Tris/HCl pH6.8, 0.4 w/v SDS)	2.5ml
Acrylamide (30% (w/v) acrylamide (37.5:1) Bisacrylamide)	1.6ml
10% Aqueous ammonium persulphate	50.0µl
TEMED	15.0µl

APPENDIX IV

Clinical, serological and HLA typing data for Bath Caucasian patients included in this study.

Patient ID	Diagnosis	Scleroderma skin type (1-3) (1+2=Ltd.)	Autoantibodies	Sex	HLA-DRB1*	HLA-DQB1*	^{71}TRAELDT^{77}	DQB1:Y:30	DQB1:Y:26	D.O.B
1	SSc, PBC	L(2)	ACA	F	0302, 0401	0604,0301/4	N,Y	N,Y	N,Y	29/09/1933
2	SSc	L(1)	ACA/ AMA	F						05/01/2016
3	SSc	D(3)	RNAP I, IIO, IIA, III	F						21/03/1958
4	SSc	L(1)	ACA	M						27/08/1943
5	SSc	L(2)	Ro, La, Jo-I, U1RNP +/-sm	F						17/07/2014
6	SSc	l(2)	Pm-Scl	F	1501-1502, 0301	0602, 0201	Y,N	Y,N	N,N	27/06/2027
7	SSc	L(1)	Topo-I, RNAP IIO, Jo-I	F	0101, 1301-1302	0501,0603/8	N,Y	N,N	N,N	14/04/2027
8	SSc	L(1)	ACA	F	0101	0501	N,N	N,N	N,N	06/05/1961
9	SSc	L(1)	ACA	F						25/08/2025
10	SSc	L(1)	RNAP I, IIO, IIA, III	F	0301, 0701-0702	0201(H)	N,N	N,N	N,N	22/11/1933
11	SSc	L(1)	ACA	F	1501-1502, 1101-1104	0602, 0301	Y,Y	Y,Y	N,Y	07/05/2005
12	SSc	D(3)	RNAP I, IIO, IIA, III	F						27/03/2017
13	SSc	L(1)	ACA	F						24/04/1937
14	SSc	L(1)	RNAP III	F						17/11/2019
15	SSc		Topo-I	F	1501-1502, 0801-0805	0602, 0301/4	Y,Y	Y,Y	N,Y	01/09/1944
16	SSc	D(3)	RNAP I, III							
17	SSc, SLE	L(1)	U1RNP +/-sm	F						14/03/1939
18	SSc	L(2)	Topo-I, Ro							09/01/1925
19	SSc	L(1)	ACA	F	0301, 0701-0702	0201, 0201	N,N	N,N	N,N	19/01/1961
20	SSc	L	Topo-I	F	0101-0102, 0701-0702	0501, 0303	N,Y	N,Y	N,N	09/01/1934
21	SSc,RA	L(1)	ACA	F	0101, 0701-0702	0501, 0201	N,N	N,N	N,N	20/04/1934
22	SSc	L(1)	ACA	F						08/06/2027

23	SSc	D(3)	Topo-I	F	1501-1502, 0301	0602, 0201	Y,N	N,Y	N,N	13/11/1935
24	SSc	L(1)	ACA	F						21/02/1935
25	SSc, RA	L(2)	Topo-I	F	0401, ?	0302, 0301	Y,Y	Y,Y	N,Y	12/04/2019
26	SSc	D(3)	Jo-I	F	0101-0102, 0301	0501, 0201	N,N	N,N	N,N	15/01/1954
27	SSc	L(1)	ACA	F						01/07/1936
28	SSc	L	ACA	F	1302, 0401	0201, 0302	N,Y	N,Y	N,N	19/11/1954
29	SSc	L(1)	ACA	F	0101, 0101	0501, 0201	N,N	N,N	N,N	06/12/2022
30	SSc	L(2)	U1RNP +/-sm	F	0401, 1301	0603/8, 0301	Y,Y	N,Y	N,Y	05/12/1953
31	SSc	L(1)	ACA	F						20/02/1949
32	SSc	L(1)	ACA	F	0101	0501	N,N	N,N	N,N	03/10/1957
33	SSc, (Hypothyroidism)	L(1)	U1RNP +sm	F						18/03/1947
34	SSc	L	ACA	F	0401-0411, 1301-1302	0603/8, 0301	Y,Y	N,Y	N,Y	22/10/2019
35	SSc	L(1)	Pos. Unid.	F	0401, 0403	0302, 0302	Y,Y	Y,Y	N,N	23/07/2027
36	SSc	L(1)	DCNS, ENA Neg.	M						28/10/2021
37	SSc	L(1)	Topo-I, Ro, La							21/07/1952
38	SSc, RA	L(1)	Ro, La, Pm-Scl	F	0301, 0801-0805	0201	N,N	N,N	N,N	12/02/1932
39	SSc	L(1)	Topo-I	F						22/03/2012
40	SSc	L(1)	ACA	F	0101, 1201-1202	0501, 0301	N,Y	N,Y	N,Y	28/12/1943
41	SSc	L(1)	Jo-I	F	0404, 1201-1202	0302, 0301/4	Y,Y	Y,Y	N,Y	08/19/25
42	SSc	L(1)	ACA	F	0401-0411, 0101-0102	0501, 0302	N,Y	N,Y	N,N	30/01/1946
43	SSc, SLE	L(1)	Jo-I, U1RNP+Sm	F	0301(H)	0201 (H)	N,N	N,N	N,N	19/03/2029
44	SSc	L(1)	ACA	F	0801-0805, 1501-1502	0602	Y,Y	Y,Y	N,N	02/19/15
45	SSc	L(1)	Topo-I	M	0301, 0404	0201, 0302	N,Y	N,Y	N,N	26/01/2027
46	SSc	L(1)	ACA	F	0101, 0404	0501, 0302	N,Y	N,Y	N,N	09/04/1931
47	SSc	D(3)	Topo-I, RNAP IIO	M						11/01/1934
48	SSc	L(1)	ACA	F	0301, 0301					08/11/1945
49	SSc	L(1)	ACA	F						28/06/2023
50	SSc	L(1)	ThRNP	F	1501-1502, 0701-0702	0602, 0201	Y,N	Y,N	N,N	14/11/1939
51	SSc		ACA	F	1501-1502, 0701-0702	0602, 0201	Y,N	Y,N	N,N	16/10/1940
52	SSc	L(2)	Jo-I	F	0301, 0901	0201, 0303	N,Y	N,Y	N,N	26/12/1951

53	SSc	L(2)	La, Pm-Scl	F	0301, 1101-1104	0201, 0301/4	N,Y	N,Y	N,Y	14/04/1956
54	SSc	D(3)	Topo-I, RNAP IIO	F	0301, 0701-0702	0201,0303	N,Y	N,Y	N,N	26/08/2022
55	SSc	L(2)	Nucleolar, ENA Neg, IP-nothing ID	F	0301, 0701-0702	0201	N,N	N,N	N,N	17/11/1951
56	SSc	D(3)	Topo-I, RNAP IIO, IIA	M	0401-0411, 1101-1104	0302, 0301	N,Y	Y,Y	N,Y	07/08/1961
57		D(3)	RNAP I, III							
58	SSc	L(1)	ACA	F	0701-0702	0201	N,N	N,N	N,N	29/10/1940
59	SSc	L(1)	ACA	F						28/01/1937
60	SSc	L(2)	ACA	F	0404, 0701-0702	0201, 0303	N,Y	N,Y	N,N	27/05/2013
61	SSc	L(1)	ACA	F	0301, 0401-0411	0201, 0302/3	N,Y	N,Y	N,N	12/09/2022
62	SSc	L(2)	RNAP I, IIO, IIA, III	F						13/04/1937
63	Raynauds	Raynauds!	ACA	F						12/01/1931
64	SSc	L(1)	ACA	F	0101-0102, 0301	0501, 0201	N,N	N,N	N,N	25/01/1952
65	SSc	L(1)	ACA	F	0101, 0101	0501 (H)	N,N	N,N	N,N	23/05/1933
66	SSc	L(1)	Topo-I, Ro, Jo-I	F	1101-1104, 1301-1302	0603/8,0301	Y,Y	N,Y	N,Y	17/11/2019
67	SSc/ diverticulitis	L(1)	ACA	F	1501-1502, 0401-0411	0602, 0302	Y,Y	Y,Y	N,N	22/08/2024
68	SSc	L(1)	Scl-70 by ENA	F	0103, 0301	0501, 0201	N,N	N,N	N,N	20/06/2010
69	SSc	L(2)	ACA	F	1501, 1502	0601,0602	Y,Y	Y,Y	Y,N	22/01/2026
70	SSc	L(1)	ACA	F						09/01/2020
71	SSc	L(2)	Topo-I	F						08/05/2020
72	SSc	D(3)	RNAP I, IIO, IIA, III	M						21/12/2020
73	SSc	L(1)	ACA	M	0301, 1101-1104	0201, 0301/4	N,Y	N,Y	N,Y	04/19/27
74	SSc	L(2)	Topo-I, RNAP IIO	F	0901, 1101-1104	0301,030	Y,Y	Y,Y	Y,Y	02/03/1951
75	SSc	L(1)	ACA	F	1501-1502, 0407	0602, 0301/4	Y,Y	Y,Y	N,Y	02/01/1943
76	SSc	L(1)	Topo-I, RNAP IIO	F	1501-1502, 0701-0702	0602, 0201	Y,N	Y,N	N,N	23/10/2019
77	SSc	L(1)	ACA	F						15/03/1949
78	SSc	D(3)	RNAP I, IIO, IIA, III							
79	SSc	L(1)	ACA	F						23/03/1957
80	SSc	L(1)	Ro, La	F	0101, 0301	0501, 0201	N,N	N,N	N,N	28/04/1934
81	SSc, RA	L(1)	Neg.	F						13/11/2026
82	SSc	L(1)	Topo-I							25/03/2018

83	SSc	L(1)	ACA	F	0101, 0401-0411	0501, 0301	N,Y	N,Y	N,Y	15/01/1933
84	SSc	D(3)	RNAP I, IIO, IIA, III	F	0301, 0408	0201, 0302	N,Y	N,Y	N,N	18/03/2019
85	SSc	L(1)	Ro	M	0101, 0301	0501, 0201	N,N	N,N	N,N	04/10/2021
86	SSc	L(1)	ACA	F	0403	0302	Y,Y	Y,Y	N,N	20/02/1931
87	SSc	L	Topo-I	M						31/08/1934
88	SSc	L(2)	Topo-I, RNAP IIO, IIA	F						10/01/1938
89	SSc	L(2)	Pos. Unid.	F	0301, 0701-0702	0201	N,N	N,N	N,N	17/09/2020
90	SSc	L(2)	Ro, La, U3RNP	M	0301, 1301	0603/8, 0201	Y,N	N,N	N,N	22/04/1942
91	SSc	D(3)	Topo-I, U1RNP+/-Sm	F	1501-1502, 1101-1104	0602, 0301	Y,Y	Y,Y	N,Y	27/06/1942
92	SSc	L(1)	ACA	F	0101, 0301	0501, 0201	N,N	N,N	N,N	10/12/2027
93	SSc	L(2)	Various ANA	F	0101, 0404	0501, 0302	N,Y	N,Y	N,N	19/02/2024
94	SSc	L(1)	ACA	F	0401-0411, 0801-0805	0302, 0302	Y,Y	Y,Y	N,N	08/12/1935
95	SSc	D(3)	RNAP IIO, Topo-I, Ro	F	1101-1104, 1302	0604, 0301/4	Y,Y	N,Y	N,Y	27/10/1940
96	SSc	L(2)	Ro, U1RNP Z	F	1501-1502, 1601-1602	0501, 0502	N,N	N,N	N,N	26/05/1949
97	SSc	D(3)	RNAP I, IIO, IIA, III	F						22/07/1939
98	SSc	D(3)	RNAP I, III	F	0701-0702	0201, 0303	N,Y	N,Y	N,N	21/08/1949
99			Topo-I		0401-0411, 0701-0702	0302, 0201	Y,N	Y,N	N,N	
100			Topo-I		1501-1502, 1101-1104	0602, 0602	Y,Y	Y,Y	N,N	

APPENDIX V:

Clinical, serological and HLA typing data for Manchester Caucasian patients included in this study.

Patient ID	Aab. Result Consensus.	ANA Pattern	Sex	Disease Subtype	HLA-DRB1*	HLA-DQB1*
SO-112	RNAP I, II, III	F. Sp. NS	F	dcSSc	0301, 04	0302, 0301
SO-230	ACA	ACA	F	lcSSc	0101, 13	0301, 05031
TO-181	ACA	ACA	M	lcSSc	0701, 1301-1302	0301, 0201/0202
MP-6	La	F. Sp. NS	F	dcSSc	0701, 13	0201/2, 0604
MP-245	Jo-1	Wk. F. Sp.	M	lcSSc	0401, 0402	0301, 0302
MP-191	ACA	ACA	F	lcSSc	0101, 0301	0201/2, 0501
BP-210	RoLa	DCNS	F	lcSSc	0901, 13	0604, 03032
BS-141	Positive Unid.	DCNS	F	lcSSc	01, 1201	0301, 0501
BS-232	Pm-Scl	Nucleolar, F. Sp.	M	lcSSc	0301, 04	0201/2, 0302
PP-162	Neg.	C. Sp. Nucleolar	F	lcSSc	1001, 11	0301, 0501
VP-106	Jo-1	Discrete cytoplasmic sp.	F	lcSSc	0301, 15	0201/2, 0603
RR-203	Pm-Scl	F. Sp. Nucleolar	F	lcSSc	NT	NT
JR-85	Topo-I	F. Sp. Nuclolar Sp.	F	dcSSc	15, 15	0602, 0602
JR-5	ACA	ACA	F	lcSSc	ND	ND
JR-61	ACA	ACA	F	lcSSc	0401, 13	0604, 0301
GR-183	Neg.	2-3 bright nuclear dots	F	lcSSc	01, 03	0501, 0603
WS-221	ACA & Positive Unid.	ACA & discrete cytoplasmic speckle	F	lcSSc	0801, 13	0402, 0604
LS-186	Ro, La	DCNS	F	lcSSc	0301, 1102	0201/2, 0301
PR-143	RNAP I,III	F. Sp. NS	F	dcSSc	04, 15	0302, 0602
MS-231	ACA	ACA	F	lcSSc	04, 15	0402, 0602
GT-219	Neg.	C. Sp. Nucleolar	M	lcSSc	13, 15	0602, 0603
IW-52	Positive Unid.	Cytoplasmic Sp. Discrete C. Sp. In nucleolus	F	lcSSc	15, 0101/02/04	0602, 0501
AT-138	ACA	ACA	F	lcSSc	0101, 0401	0501, 0301/2

MS-255	U1RNP	F. Sp. NS	F	lcSSc	0301, 04	0201/2, 0302
IS-260	ACA	ACA	F	lcSSc	0101, 04	0301, 0501
PS-239	ACA	ACA	F	lcSSc	04, 11	0301, 0302
JW-184	ACA	ACA	F	lcSSc	0310, 0801	0201/2, 0402
AW-241	Positive Unid.	Nucleolar, C. Sp.	F	lcSSc	0301, 14	0201/2, 05013
PS-187	ACA	ACA	F	lcSSc	0701, 04	0201/0202, 03032/0302
CW-142	Positive Unid.	F. Sp.	F	dcSSc	ND	ND
JS-144	Pm-Scl	F. Sp.	F	lcSSc	0701, 1301/1302	0201/0202, 0201/0202
GW-213	Topo-I	Homogeneous	M	dcSSc	0101/0102/0104, 0301	0501, 0201/0202
IT-22	Jo-1	F. Sp. NS	F	lcSSc	15, 0301	0602, 0201/0202
AW-180	ACA	ACA	F	lcSSc	04, 14	05031, 03
LW-205	Positive Unid.	3-4 patches of tiny dots.	F	lcSSc	0402, 0404	0302, 0302
MT-16	Neg.	Cytoplasmic Sp.	F	LcSSc & Overlap	ND	ND
CT-18	Neg.	DCNS	F	lcSSc	0701, 1301/1302/1402/1406/14 09/1413/1417	0301, 0201/0202
DY-248	Neg.	C. Sp. NS	M	dcSSc	0101, 15	0501, 0602
DW-200	Neg.	Wk. F. Sp. (Neg)	F	lcSSc	0301, 0701	0201/2, 03032
KT-135	Positive Unid.	A mess, failed.	F	lcSSc	0101, 0701	0501, 0201/2
ET-79	ACA	ACA	F	lcSSc	NT	0301, 0302
CW-246	La	F. Sp. NS	F	dcSSc	0301, 0701	0201/2, 0201/2
LT-199	U1RNP	DCNS	F	lcSSc	0301, 0401	0201/2, 0301
BT-193	ACA (wk)	ACA	F	lcSSc	1001, 1101	0301, 0501
SC-4	wk. Topo-I	Wk. F. Sp. (Neg)	M	lcSSc	0101, 11	0301, 0501
SC-280	Neg.	Homogeneous	F	lcSSc	15, 04	0302, 0602
SC-72	ACA	ACA	F	lcSSc	15, 11	0301, 0602
SC-95	Neg.	Wk. F. Sp.	F	dcSSc	0701, 0701	0201/2, 03032
SC-274	Topo-I	F. Sp. Nucleolar	F	dcSSc	0103, 15	0301, 0602
SC-197	ACA	ACA	F	lcSSc	0101, 0301	0201/2, 0501
AH-188	RNAP I, II, III	Nucleolar, C. Sp.	M	dcSSc	0403, 0404	0302, 0304
AB-26	ACA	ACA	F	lcSSc	NT	NT

MC-27	ACA	ACA	F	lcSSc	15, 0301	0602, 0201/0202
BD-272	RNAP I, IIwk, III	Clumpy Nucleolar, discrete cytoplasmic dots.	F	dcSSc	0101, 0301	0501, 0201/2
JF-87	Pm-Scl	F. Sp. Nucleolar	F	lcSSc	0301, 13	0201/2, 0604
MG-267	Positive Unid.	Homogeneous.	F	lcSSc	0701, 04	0302, 03032
FC-204	ACA	ACA	F	lcSSc	11, 13	0301, 0604
KM-220	U1RNP	DCNS	M	lcSSc	0301, 0401	0201/2, 0301
JM-177	ACA	ACA (wk)	F	dcSSc	NT	0301, 0301
AC-137	ACA	ACA	F	lcSSc	0101, 04	0302, 0501
TB-224	Ro, La	F. Sp. NS	M	dcSSc	0301, 11	0201/2, 0301
LH-146	ACA	ACA	F	lcSSc	0102, 0401	0501, 0302
WD-262	U1RNP	DCNS	F	lcSSc	0301, 0401	0201/2, 0302
KF-192	U1RNP	DCNS	M	lcSSc	0101, 0301	0201/2, 0501
JG-194	ACA	ACA	F	lcSSc	0801, 1101	0301, 0402
AH-71	ACA	ACA	F	lcSSc	0101, 0101	0501, 0501
SJ-244	ACA	ACA	F	dcSSc	0801, 13	0402, 0604
SM-70	RNAP II, Topo-I	F. Sp. Nucleolar	F	lcSSc	0101, 1104	0301, 0501
AF-202	Failed sample (Hint of Pm-scl but not confirmed)	F. Sp	F	lcSSc	NT	NT
SB-25	ACA	ACA	F	lcSSc	04, 04	0301, 0301
JC-268	ACA	ACA	F	lcSSc	0701, 15	0201/2, 0602
MD-43	RNAP I, II, III	F. Sp. Nucleolar	F	dcSSc	0101, 1301	0501, 0603
PF-263	Neg.	(wk) F. Sp. Nucleolar	F	lcSSc	15, 0701	0602, 0201/0202
GG-265	ACA	ACA	F	lcSSc	0101, 0801	0402, 0501
GH-247	Failed sample (Hint of Pm-scl but not confirmed)	Neg	F	lcSSc	15, 0301	0201/2, 0602
CK-129	RNAP I, IIwk, III	F. Sp.	F	dcSSc	0103, 0103	0501, 0501
MM-217	RNAP I, III	F. Sp.	F	lcSSc	0101, 0701	0201/2, 0501
AG-179	Positve Unid.	Clumpy Nucleolar & C. Cytoplasmic Sp.	F	lcSSc	15, 0401	0302, 0602
FM-257	ACA (wk)	ACA (wk)	M	lcSSc	04, 04	0301, 0302
JC-233	Neg.	Neg	F	lcSSc	0301, 0701	0201/2, 0201/2
BF-264	ACA	ACA	F	lcSSc	0101/0102/0104,	0501, 0302/03031/03032

					0401/0403-0413	
KG-145	Topo-I	Homozygous	F	dcSSc	NT	NT
JH-28	Multiple Unid. Bands	F. Sp. Wk Nucleolar	M	dcSSc	11, 1103	0301, 0301
BK-110	Neg.	Neg.	M	dcSSc	NT	NT
LM-97	Neg.	Neg.	F	lcSSc	13, 14	05031, 0604
JB-78	Topo-I	F. Sp. Nucleolar	M	dcSSc	04, 1301/1302	0301, 0301
CB-89	ACA	ACA	F	lcSSc	0102, 0401	0302, 0501
DC-196	ACA	ACA	F	lcSSc	0101, 15	0501, 0602
VE-36	RNAP I,II,III	C. Sp. NS	F	dcSSc	11, 14	0301, 05031
HF-269	wk U1RNP	DCNS	F	lcSSc	0301, 0401	0201/2, 0302
PH-46	Positive Unid.	C. Cytoplasmic Sp.	F	lcSSc	04, 03	0201/0202, 0302/03031/03032
JH-136	ACA	ACA, F. Sp.	F	lcSSc	0103, 13	0501, 0605
ML-211	Positive Unid.	Neg.	F	dcSSc	0301, 0701	0201/2, 03032
AM-86	wk. Unidentified	Neg.	M	lcSSc	04, 04	0301, 0302
BB-132	RNAP I, II, III	Clumpy Nucleolar	F	lcSSc	0301, 071	0201/2, 0201/2
BC-256	Ro, La, U3RNP	Clumpy Nucleolar	F	lcSSc	0301, 0801	0402, 0201
JB-233	U1RNP	DCNS	F	lcSSc	0101, 0401	0302, 0501
ME-190	Topo-I	Strong F. Sp.	F	dcSSc	0101, 0701	0501, 03032
SF-74	ACA	ACA	F	lcSSc	01011/0102/0104, 04	0501, 0301
GH-228	U1RNP	DCNS	M	lcSSc	0301-0302, 0303	0201/0202, 0201/0202
PH-240	ACA (wk)	Wk ACA	F	lcSSc	0103, 0701	0501, 0201/0202
BL-21	ACA	ACA	M	lcSSc	04, 0301	0301, 0201/0202
JM-175	RNAP I, IIwk, III	Wk DCNS	F	dcSSc	0103, 15	0602, 0301
GB-139	Pm-Scl	Clumpy Nucleolar	F	lcSSc	0301, 15	0201/2, 0603
CB-118	Topo-I	Nucleolar.	F	dcSSc	0301, 13	0201/2, 0604
TC-222	ACA	ACA	F	lcSSc	NT	0302, 0501
PF-266	ACA	ACA	F	lcSSc	0101, 13	0501, 0603
BG-218	Topo-I	F. Sp. Nucleolar.	F	dcSSc	04, 1103	0301, 0302
RH-107	Neg.	Neg.	F	lcSSc	0301, 0401	0201/2, 0301
H-31	ACA	ACA	M	lcSSc	0101/0102/0104, 0403-13/0417-19	0403-13/0417-19

AL-34	RNAP I,II,III	F. Sp. NS.	F	dcSSc	0103, 11	0301, 0301
DM-17	Positive Unid.	Positive unid.	F	SSc	0301, 04	0201/2, 0302
LB-249	U1RNP	DCNS	F	lcSSc	nt	0301, 0302
DB-133	Ro, Pm-Scl	F. Sp. Nucleolar.	F	lcSSc	0301, 04	0201/2, 0302
DD-227	Positive Unid.	F. Sp. NS	M	lcSSc	NT	0603, 0604
RF-209	Topo-I	F. Sp. Nucleolar.	M	dcSSc	15, 0101/0102/0104	0602, 0501
CG-206	ACA	ACA	F	lcSSc	0101/0102/0104, 03	0501, 0201/0202
SH-212	Pm-Scl	F. Sp. Nucleoalar.	F	lcSSc	0701, 0301	0201/2, 0201/2
BH--69	ACA	ACA	F	lcSSc	0701, 01	0501, 0201/0202
MM-195	ACA	ACA	F	lcSSc	04, 13	0302, 0603
AB-270	RNAP I,II,III	DCNS	M	dcSSc	04, 08	0402, 03
JD-66	RNAP I,II,III	f. Sp. NS.	F	dcSSc	0301, 0301	0201/0202, 0201/0202
MB-251	Neg.	Neg.	F	dcSSc	2(DR15), 2(DR16)	0602, 06011/06012
BF-216	V. wk. Topo-I	F. Cytoplasmic Sp. & F. Sp.	M	dcSSc	0101, 15	0501, 0602
PG-229	ACA	ACA	F	lcSSc	0301, 04	0201/2, 0302
JH-140	ACA	ACA	F	lcSSc	04, 08	0302, 0402
NH-253	ACA	ACA	F	lcSSc	15, 0401/0409/0412/0413/04 18	0602, 0302/03032
YM-167	ACA	ACA	F	lcSSc	15, 0301	0602, 0201/0202
TN-149	Neg.	Nucleolar	F	lcSSc	0301, 0701	0201/2, 0201/2

APPENDIX VI:

Clinical, serological and genetic data for South African Black SSc patients included in this study

Patient ID	Sex	Age	Disease Subtype	PF	Aab Result Consensus	Ouchterlony	Indirect Immunofluorescence	HLA DRB1*	HLA DQB1*
D2	F	49	dcSSc	N	Positive Unidentified	No Line	Homozygous	1102, 1302	0311/12/04
D3	F	47	lcSSc	N	Positive Unidentified	Not Ro or La	F.Sp. V.Wk 1/40	1101, 1301	0604-9/12, 0603/14
D4	M	38	lcSSc	Y	Wk. Positive unidentified, (possible V. Wk. U1RNP)	No Line	DCNS	1001, 1501	0501-0504, 0602/10/11
D5	F	54	lcSSc	N	Wk. Positive unidentified, (possible V. Wk. U1RNP)	No Line	DCNS	0301, 1102	0201-0203, 0201-0203
D8	F	50	lcSSc	N	U3RNP		F. Sp. & Sp. Nucleolar	1101, 1101	0602/ 10/ 11, 0602/ 10/ 11
D9	F	17	dcSSc	N	Negative		Wk. Homozygous	0302, 0302	0401-0402, 0401-0402
D11	F	26	lcSSc	N	Ro		F. Sp. 1/40	0301, 1001	0201-0203, 0501-0504
D13	F				ACA		ACA	07, 1301	0201-0203, 0603/4
D14	F	40	dcSSc	N	ACA		ACA	1302, 1501	0602-4/ 9-12/ 14 (H)
D15	F	43	dcSSc	N	U3RNP & Positive Unidentified	No Line	Nucleolar Clumpy	1101, 1201	03011/12/04, 0501-0504
D16	F				U3RNP		Nucleolar Clumpy	1101, 1301	03011/12/04, 0603/4
D17	F	42	dcSSc	N	RNAP I, III & Ro	Ro	F. Sp. & Nucleolar Clumpy	0302, 1501	0401-0402, 0602/10/11
D18	F	48	lcSSc	N	ACA		ACA	1301, 1301	0602-4/ 9-12/ 14 (H)
D19	F	38	dcSSc	Y	Topo-I	Topo-I	F. Sp	1101, 1101	03011/12/04, 0602/10/11
D21	F	47	lcSSc	N	Positive Unidentified		F. Sp. NS	0302, 1301	0401-0402, 0604-9/12
D22					Ro, La		DCNS	08, 1501	0311/12/04, 0602/10/11
D23	F	28	dcSSc	Y	Ro	Ro	DCNS	0302, 1501	0401-0402, 0602/10/11
D25	F	37	lcSSc	N	Ro, La, U1RNP	U1RNP, Ro, (*Blot:* La, U1RNP)	DCNS	0301, 1001	0201-0203, 0501-0504
D26	F	42	lcSSc	N	Positive Unidentified	No Line	DCNS	0301, 07	0201-0203, 0201-0203
D28	F	50	dcSSc	Y	Topo-I	Topo-I	F. Sp & Nucleolar	0302, 1201	0401-0402, 0501-0504

D29	F	47	dcSSc	N	ACA & Positive Unidentified		ACA & F. Sp.	0102/4, 0102/4	0501-0504, 0501-0504
D31	F	37	dcSSc	N	Weak Positive Unidentified		Wk. F. Sp. NS	1102, 1301	0311/12/04, 0602/10/11
D32	M	40	dcSSc	N	Ro, La, U1RNP, Topo-I		DCNS	07, 1301	0401-0402, 0602/10/11
D33	F	66	lcSSc	N	U1RNP +/-sm	U1RNP	DCNS	0302, 1501	0401-0402, 0602/10/11
D34	M	44	lcSSc	N	Ro, U1RNP +/-sm	Ro	F. Sp. & Cytoplasmic Sp. & Cytoskeletal & Nuclear Rim	0301, 07	0201-0203, 0201-0203
D35	F	40	dcSSc	N	U3RNP & Positive Unidentified	Pos. unid	Nucleolar Clumpy	04, 1301	0401-0402, 0603/4
D37	F	31	lcSSc	N	Negative		Wk. F. Sp. & Wk. Cytoplasmic	0102/4, 1501	0501-0504, 0602/10/11
D38	F	59	dcSSc	Y	Positive unidentified	Pm-Scl	Nucleolar Clumpy	1201, 0302	0401-0402, 0501-0504
D39	F	54	dcSSc	N	Ro, La	Not Jo-1	F. Sp. NS	04, 1302	0401-0402, 0604-9/12
D40	F	33	dcSSc	N	Positive Unidentified	No Line	Nucleolar Clumpy	08, 1301	0311/12/04, 0501-0504
D41	M	47	dcSSc	N	Positive Unidentified		F. Sp.	0102/4, 1301	0501-0504, 0604-9/12
D42	F	58	lcSSc	N	U3RNP		F. Sp.	1201, 1301	0501-0504, 0603/14
D43	F	33	lcSSc	N	Topo-I	V. Wk pos. (unid'd)	Nucleolar & Discrete Nuclear Sp.	1001, 1501	0501-0504, 0602/10/11
D44	F	18	dcSSc	N	Topo-I	Topo-I	F. Sp.	1301, 1501	0602-4/ 9-12/ 14 (H)
D45	F	39	dcSSc	N	Negative		Nucleolar & Discrete Sp.	1102, 1501	03011/12/04, 0602/10/11
D47	F	46	dcSSc	N	RNAP IIo, Topo-I		F. Sp.	1301, 1501	0602-4/ 9-12/ 14
D48	F	63	dcSSc	N	RNAP I, III		F. Sp Nucleolar & F. Sp.	1101, 1301	03011/12/04 0604-9/12
D49	F	32	dcSSc	N	Weak Positive Unidentified	No Line	Nucleolar.		
D51	F	41	dcSSc		V. Wk. U1RNP +/-sm & V. Strong Unidentified postitive.	Wk. U1RNP & strong unid.	Cytoplasmic & Nucleolar.	0301, 0301	0201-0203, 0201-0203
D52	F	40	dcSSc	N	U1RNP +/-sm	U1RNP	F. Sp. & Nucleolar	0102/4 1201	0501-0504, 0501-0504
D53	F	37	dcSSc	N	U1RNP +/-sm	U1RNP	DCNS	04, 1401/2	03011/12/04, 0602/10/11
D54	F	36	lcSSc		U3RNP		Nucleolar Clumpy		
D56	F	55	dcSSc	N	U3RNP		Nucleolar Clumpy		
D57	F	54	dcSSc	N	U1RNP +/-sm	Possible U1RNP	DCNS		
D58	M	38	dcSSc		U3RNP		Nucleolar Clumpy		

APPENDIX VII:

HLA-DRB1* and HLA-DQB1* typing data for 250 British Caucasian normal controls, with reference of amino acids at specific loci considered in this work.

ID Number	HLA-DRB1*	HLA-DQB1*	HLA-DQB1:^{71}TRAEDLT^{77}	HLA-DQB1:30:Y?	HLA-DQB1:26	HLA-DQB1:26:Y?
1	0101, 0701-0702	0201, 0501	N, N	N/N	L,G	N,N
2	0301, 1401/1404-1405	0503, 0201	N, N	N/N	G,L	N,N
3	0401, 1301-1302	0603/8, 0302	Y, Y	N/Y	L,L	N,N
4	0301, 1501-1502	0602, 0201	Y, N	Y/N	L,L	N,N
5	1501-1502,1101-1104	0602, 0301/4	Y, Y	Y/Y	L,T	N,Y
6	0301, 0701-0702	0201 (H)	N, N	N/N	L,L	N,N
7	0102 (H)	0501 (H)	N, N	N/N	G,G	N,N
8	0101, 0901	0501, 0303	N, Y	N/Y	G,L	N,N
9	1501-1502, 0301	0602, 0201	Y, N	Y/N	L,L	N,N
10	0301, 0401	0201, 0301/4	N, Y	N/Y	L,T	N,Y
11	1501-1502, 0401	0602, 0301/4	Y, Y	Y/Y	L,T	N,Y
12	0401, 0407	0301/4 (H)	Y, Y	Y/Y	T,T	Y,Y
13	0301, 0403	0201, 0301/4	N, Y	N/Y	L,T	N,Y
14	1501-1502, 0701-0702	0602, 0201	Y, N	Y/N	L,L	N,N
15	0801-0805, 1101-1104	0301/4 (H)	Y, Y	Y/Y	T,T	Y,Y
16	0301, 0701-0702	0201 (H)	N, N	N/N	L,L	N,N
17	0401, 1302	0604, 0301/4	Y, Y	N/Y	L,T	N,Y
18	1501-1502, 0401	0602, 0301/4	Y, Y	Y/Y	L,T	N,Y
19	0401, 0701-0702	0302, 0303	Y, Y	Y/Y	L,L	N,N
20	0801-0805, 0901	0601, 0303	Y, Y	Y/Y	T,L	Y,Y
21	1601-1602, 0701-0702	0502, 0201	N, N	N/N	G,L	N,N
22	1501-1502, 0701-0702	0602, 0201	Y, N	Y/N	L,L	N,N

23	0401, 0701-0702	0201, 0301/4	N, Y	N/Y	L,T	N,Y
24	0102, 1501-1502	0501, 0502	N, N	N/N	G,G	N,N
25	1601-1602, 0401	0502, 0301/4	N, Y	N/Y	G,T	N,Y
26	1302, 0404	0602, 0302	Y, Y	Y/Y	L,L	N,N
27	1501-1502 (H)	0602 (H)	Y, Y	Y/Y	L,L	N,N
28	1501-1502, 0401	0602, 0302	Y, Y	Y/Y	L,L	N,N
29	0101, 1101-1104	0501, 0301/4	N, Y	N/Y	G,T	N,Y
30	1501-1502, 0701-0702	0601, 0303	Y, Y	Y/Y	T,L	Y,Y
31	0701-0702, 1302	0604, 0303	Y, Y	N/Y	L,L	N,N
32	1302 (H)	0604 (H)	Y, Y	N/N	L,L	N,N
33	1101-1104 (H)	0601, 0301/4	Y, Y	Y/Y	T,T	Y,Y
34	0407, 0301	0201, 0301/4	N, Y	N/Y	L,T	N,Y
35	1601-1602, 0801-0805	0502, 0402	N, N	N/Y	G,G	N,N
36	0404, 1301-1302	0603/8, 0302	Y, Y	N/Y	L,L	N,N
37	0301 (H)	0201 (H)	N, N	N/N	L,L	N,N
38	0101, 1302	0501, 0604	N, Y	N/N	G,L	N,N
39	0404, 1302	0604, 0302	Y, Y	N/Y	L,L	N,N
40	1501-1502, 1101-1104	0602, 0301/4	Y, Y	Y/Y	L,T	N,Y
41	1501-1502, 1301-1302	0602, 0603/8	Y, Y	Y/N	L,L	N,N
42	1401/4/5, 0701-0702	0201, 0503	N, N	N/N	L,G	N,N
43	1501-1502, 1303-1304	0602, 0301/4	Y, Y	Y/Y	L,T	N,Y
44	0801-0805 (H)	0402 (H)	N, N	Y/Y	G,G	N,N
45	0101, 0701-0702	0501, 0303	N, Y	N/Y	G,L	N,N
46	0101, 0301	0501, 0201	N, N	N/N	G,L	N,N
47	0701-0702, 0404	0201, 0302	N, Y	N/Y	L,L	N,N
48	0701-0702 (H)	0303 (H)	Y, Y	Y/Y	L,L	N,N
49	1501-1502, 0301	0602, 0201	Y, N	Y/N	L,L	N,N
50	0701-0702, 1301-1302	0303, 0603/8	Y, Y	Y/N	L,L	N,N
51	1501-1502, 1302	0602, 0604	Y, Y	Y/N	L,L	N,N
52	1501-1502, 1302	0602, 0604	Y, Y	Y/N	L,L	N,N
53	1501-1502, 1302	0602,0603/8	Y, Y	Y/N	L,L	N,N

54	1501-1502, 1302	0602 (H)	Y, Y	Y/Y	L,L	N,N
55	0401, 0901	0301/4, 0303	Y, Y	Y/Y	T,L	Y,N
56	0301, 1101-1104	0201, 0301/4	N, Y	N/Y	L,T	N,Y
57	1501-1502, 0301	0602, 0201	Y, N	Y/N	L,L	N,N
58	0701-0702, 1001	0501, 0201	N, N	N/N	G,L	N,N
59	1501-1502, 0301	0602 (H)	Y, Y	Y/Y	L,L	N,N
60	0301, 1302	0201, 0302	N, Y	N/Y	L,L	N,N
61	0301, 0801-0805	0201, 0402	N, N	N/Y	L,G	N,N
62	0101, 1101-1104	0501, 0301/4	N, Y	N/Y	G,T	N,Y
63	0301, 0101	0502, 0201	N, N	N/N	G,L	N,N
64	0301 (H)	0201 (H)	N, N	N/N	L,L	N,N
65	1501-1502, 1302	0602, 0604	Y, Y	Y/N	L,L	N,N
66	0701-0702, 1302	0604, 0201	Y, N	N/N	L,L	N,N
67	1302, 0801	0604, 0402	Y, N	N/Y	L,G	N,N
68	0701-0702, 1302	0303, 0604	Y, Y	Y/N	L,L	N,N
69	1501-1502, 0407	0602, 0301/4	Y, Y	Y/Y	L,T	N,Y
70	1501-1502, 0405	0602, 0201	Y, N	Y/N	L,L	N,N
71	0401, 0701-0702	0303, 0302	Y, Y	Y/Y	L,L	N,N
72	0401, 0801-0805	0301/4, 0402	Y, N	Y/Y	T,G	Y,N
73	0101, 0701-0702	0501, 0201	N, N	N/N	G,L	N,N
74	0404, 1101-1104	0301/4, 0302	Y, Y	Y/Y	T,L	Y,N
75	0101, 1301	0501, 0603/8	N, Y	N/N	G,L	N,N
76	0701-0702 (H)	0603/8, 0303	Y, Y	N/Y	L,L	N,N
77	0101 (H)	0501 (H)	N, N	N/N	G,G	N,N
78	0401, 0301	0201, 0302	N, Y	N/Y	L,L	N,N
79	0401, 1101-1104	0301/4 (H)	Y, Y	Y/Y	T,T	Y,Y
80	1101-1104, 1302	0604, 0301/4	Y, Y	N/Y	L,T	N,Y
81	1301 (H)	0603/8 (H)	Y, Y	N/N	L,L	N,N
82	0301 (H)	0201 (H)	N, N	N/N	L,L	N,N
83	1501-1502, 1101-1104	0602, 0301/4	Y, Y	Y/Y	L,T	N,Y
84	0101, 1401/4/5	0501, 0503	N, N	N/N	G,G	N,N

85	0408, 1101-1104	0301/4 (H)	Y, Y	Y/Y	T,T	Y,Y
86	0101, 0301	0501, 0201	N, N	N/N	G,L	N,N
87	0101, 0401	0501, 0301/4	N, Y	N/Y	G,T	N,Y
88	0701-0702, 0301	0201 (H)	N, N	N/N	L,L	N,N
89	0401, 1302	0604, 0302	Y, Y	N/Y	L,L	N,N
90	0407, 0701-0702	0201, 0301/4	N, Y	N/Y	L,T	N,Y
91	0401, 0701-0702	0302, 0201	Y, N	Y/N	L,L	N,N
92	0401 (H)	0302 (H)	Y, Y	Y/Y	L,L	N,N
93	0101, 1101-1104	0501, 0301/4	N, Y	N/Y	G,T	N,Y
94	0101, 1101-1104	0501, 0301/4	N, Y	N/Y	G,T	N,Y
95	1501-1502, 0301	0602, 0201	Y, N	Y/N	L,L	N,N
96	0404 (H)	0302 (H)	Y, Y	Y/Y	L,L	N,N
97	0101, 1201-1202	0501, 0301/4	N, Y	N/Y	G,T	N,Y
98	0401, 1302	0604, 0302	Y, Y	N/Y	L,L	N,N
99	1501-1502 (H)	0602 (H)	Y, Y	Y/Y	L,L	N,N
100	0301, 1101-1104	0201, 0301/4	N, Y	N/Y	L,T	N,Y
101	0401 (H)	0301/4 (H)	Y, Y	Y/Y	T,T	Y,Y
102	0701-0702 (H)	0602, 0302	Y, Y	Y/Y	L,L	N,N
103	0404, 1302	0604, 0302	Y, Y	N/Y	L,L	N,N
104	1501-1502, 0701-0702	0602, 0303	Y, Y	Y/Y	L,L	N,N
105	0101, 1301-1302	0501, 0603/8	N, Y	N/N	G,L	N,N
106	0701-0702, 0401	0301/4, 0303	Y, Y	Y/Y	T,L	Y,N
107	0101, 0301	0201, 0501	N, N	N/N	L,G	N,N
108	0408, 1501-1502	0602, 0301/4	Y, Y	Y/Y	L,T	N,Y
109	0701-0702 (H)	0201 (H)	N, N	N/N	L,L	N,N
110	0404, 1101-1104	0301/4, 0302	Y, Y	Y/Y	T,L	Y,N
111	0101, 1101-1104	0501, 0301/4	N, Y	N/Y	G,T	N,Y
112	0101, 1301-1302	0603/8, 0501	Y, N	N/N	L,G	N,N
113	1501-1502, 0701-0702	0602, 0201	Y, N	Y/N	L,L	N,N
114	1501-1502, 0301	0602, 0201	Y, N	Y/N	L,L	N,N
115	1601-1602, 0301	0502, 0201	N, N	N/N	G,L	N,N

116	0801-0805, 1201-1202	0602, 0301/4	Y, Y	Y/Y	L,T	N,Y
117	1501-1502, 1301	0602, 0603/8	Y, Y	Y/N	L,L	N,N
118	1501-1502, 0701-0702	0303, 0602	Y, Y	Y/Y	L,L	N,N
119	1501-1502, 1101-1104	0602, 0303	Y, Y	Y/Y	L,L	N,N
120	0701-0702 (H)	0302, 0201	Y, N	Y/N	L,L	N,N
121	0701-0702 (H)	0201 (H)	N, N	N/N	L,L	N,N
122	1501-1502, 1301	0602, 0603/8	Y, Y	Y/N	L,L	N,N
123	1101-1104, 1303-1304	0301/4 (H)	Y, Y	Y/Y	T,T	Y,Y
124	0101 (H)	0501 (H)	N, N	N/N	G,G	N,N
125	1501-1502, 0301	0602, 0201	Y, N	Y/N	L,L	N,N
126	1501-1502, 0301	0603/8, 0201	Y, N	N/N	L,L	N,N
127	0101, 1301-1302	0201, 0302	N, Y	N/Y	L,L	N,N
128	0404 (H)	0302, 0301/4	Y, Y	Y/Y	L,T	N,Y
129	0301, 1101-1104	0201, 0301/4	N, Y	N/Y	L,T	N,Y
130	0701-0702, 1101-1104	0301/4, 0303	Y, Y	Y/Y	T,L	Y,N
131	0701-0702, 0401	0303, 0302	Y, Y	Y/Y	L,L	N,N
132	0101, 0401	0501, 0302	N, Y	N/Y	G,L	N,N
133	0101, 0401	0501, 0301/4	N, Y	N/Y	G,T	N,Y
134	1301, 0404	0603/8, 0302	Y, Y	N/Y	L,L	N,N
135	0404 (H)	0301/4, 0302	Y, Y	Y/Y	T,L	Y,N
136	1501-1502, 0301-0302	0602, 0201	Y, N	Y/N	L,L	N,N
137	1501-1502 (H)	0602 (H)	Y, Y	Y/Y	L,L	N,N
138	0401, 1501-1502	0604, 0301/4	Y, Y	N/Y	L,T	N,Y
139	0401, 0404	0302, 0301/4	Y, Y	Y/Y	L,T	N,Y
140	0701-0702, 1101-1104	0301/4, 0303	Y, Y	Y/Y	T,L	Y,N
141	1101-1104, 1201-1202	0301/4 (H)	Y, Y	Y/Y	T,T	Y,Y
142	0101, 1302	0501, 0604	N, Y	N/N	G,L	N,N
143	0101, 0701-0702	0501, 0201	N, N	N/N	G,L	N,N
144	0301, 0402	0201, 0302	N, Y	N/Y	L,L	N,N
145	1302, 1101-1104	0604, 0301/4	Y, Y	N/Y	L,T	N,Y
146	0701-0702, 1501-1502	0201, 0602	N, Y	N/Y	L,L	N,N

147	0101, 1501-1502	0501, 0602	N, Y	N/Y	G,L	N,N
148	0103, 0401	0501, 0301/4	N, Y	N/Y	G,T	N,Y
149	1501-1502, 0101	0501, 0602	N, Y	N/Y	G,L	N,N
150	1601-1602, 0701-0702	0502, 0201	N, N	N/N	G,L	N,N
151	0401, 0701-0702	0201, 0301/4	N, Y	N/Y	L,T	N,Y
152	1302 (H)	0603/8, 0604	Y, Y	N/N	L,L	N,N
153	0401, 0404	0301/4, 0302	Y, Y	Y/Y	T,L	Y,N
154	0301, 0401	0201, 0302	N, Y	N/Y	L,L	N,N
155	0701-0702, 1301	0603/8, 0201	Y, N	N/N	L,L	N,N
156	0401, 0701-0702	0201, 0301/4	N, Y	N/Y	L,T	N,Y
157	0101, 1501-1502	0501, 0602	N, Y	N/Y	G,L	N,N
158	0301 (H)	0201 (H)	N, N	N/N	L,L	N,N
159	0801-0805 (H)	0402 (H)	N, N	Y/Y	G,G	N,N
160	1302, 0701-0702	0604, 0201	Y, N	N/N	L,L	N,N
161	0701-0702, 1301	0603/8, 0201	Y, N	N/N	L,L	N,N
162	0101, 1302	0501, 0604	N, Y	N/N	G,L	N,N
163	0102, 0401	0501, 0302	N, Y	N/Y	G,L	N,N
164	1501-1502, 1101-1104	0604, 0301/4	Y, Y	N/Y	L,T	N,Y
165	1501-1502, 0901	0603/8, 0303	Y, Y	N/Y	L,L	N,N
166	1501-1502, 1301	0603/8 (H)	Y, Y	N/N	L,L	N,N
167	0101, 1501-1502	0501, 0602	N, Y	N/Y	G,L	N,N
168	0701-0702 (H)	0201 (H)	N, N	N/N	L,L	N,N
169	0101, 0401	0501, 0301/4	N, Y	N/Y	G,T	N,Y
170	0301, 0401	0201, 0302	N, Y	N/Y	L,L	N,N
171	0301, 1302	0604, 0201	Y, N	N/N	L,L	N,N
172	0301, 1302	0604, 0201	Y, N	N/N	L,L	N,N
173	0301, 0401	0201, 0301/4	N, Y	N/Y	L,T	N,Y
174	1101-1104 (H)	0301/4	Y, Y	Y/Y	T,T	Y,Y
175	0401 (H) (0408?)	0302, 0301/4	Y, Y	Y/Y	L,T	N,Y
176	0101, 0701-0702	0501, 0303	N, Y	N/Y	G,L	N,N
177	0101, 0404	0302, 0501	Y, N	Y/N	L,G	N,N

178	1101-1104, 0401	0301/4 (H)	Y, Y	Y/Y	T,T	Y,Y
179	0701-0702 (H)	0201 (H)	N, N	N/N	L,L	N,N
180	0301, 1101-1104	0201, 0301/4	N, Y	N/Y	L,T	N,Y
181	1501-1502, 1101-1104	0602, 0301/4	Y, Y	Y/Y	L,T	N,Y
182	1101-1102, 0701-0702	0301/4, 0303	Y, Y	Y/Y	T,L	Y,N
183	1101-1104, 0404	0302, 0301/4	Y, Y	Y/Y	L,T	N,Y
184	0404, 0301	0201, 0302	N, Y	N/Y	L,L	N,N
185	1101-1104, 1201-1202	0301/4 (H)	Y, Y	Y/Y	T,T	Y,Y
186	0301 (H)	0201 (H)	N, N	N/N	L,L	N,N
187	0103, 1501-1502	0501, 0602	N, Y	N/Y	G,L	N,N
188	0301 (H)	0201 (H)	N, N	N/N	L,L	N,N
189	0401, 1501-1502	0602, 0302	Y, Y	Y/Y	L,L	N,N
190	1501-1502, 0301	0201, 0602	N, Y	N/Y	L,L	N,N
191	0701-0702, 1101-1104	0201, 0301/4	N, Y	N/Y	L,T	N,Y
192	0401, 0801-0805	0301/4, 0402	Y, N	Y/Y	T,G	N,Y
193	0301, 0402	0302, 0201	Y, N	Y/N	L,L	N,N
194	1501-1502, 1001	0501, 0602	N, Y	N/Y	G,L	N,N
195	0401, 0701-0702	0303, 0301/4	Y, Y	Y/Y	L,T	N,Y
196	0401, 1301-1302	0603/8, 0301/4	Y, Y	N/Y	L,T	N,Y
197	0701-0702, 1101-1104	0303, 0301/4	Y, Y	Y/Y	L,T	N,Y
198	0401, 0701-0702	0201, 0301/4	N, Y	N/Y	L,T	N,Y
199	1501-1502, 1101-1104	0602, 0301/4	Y, Y	Y/Y	L,T	N,Y
200	0701-0702, 0408	0301/4, 0303	Y, Y	Y/Y	T,L	Y,N
201	0101, 0301	0201, 0501	N, N	N/N	L,G	N,N
202	0407, 0401	0302, 0301/4	Y, Y	Y/Y	L,T	N,Y
203	0101, 1401/4/5	0503, 0501	N, N	N/N	G,G	N,N
204	0401,1401/4/5	0301/4, 0503	Y, N	Y/N	T,G	Y,N
205	1501-1502, 0801-0805	0402, 0602	N, Y	Y/Y	G,L	N,N
206	1501-1502 (H)	0603/8, 0602	Y, Y	N/Y	L,L	N,N
207	0407, 1601-1602	0502, 0301/4	N, Y	N/Y	G,T	N,Y
208	0101, 1301-1302	0501, 0604	N, Y	N/N	G,L	N,N

209	0103, 1501-1502	0602, 0301/4	Y, Y	Y/Y	L,T	N,Y
210	0301, 1302	0201, 0603/8	N, Y	N/N	L,L	N,N
211	0401, 0101	0501, 0301/4	N, Y	N/Y	G,T	N,Y
212	0701-0702, 1201-1202	0301/4, 0303	Y, Y	Y/Y	T,L	Y,N
213	1501-1502, 1101-1104	0602, 0301/4	Y, Y	Y/Y	L,T	N,Y
214	0404, 0101	0501, 0302	N, Y	N/Y	G,L	N,N
215	0102, 1201-1202	0501, 0301/4	N, Y	N/Y	G,T	N,Y
216	0301, 1302	0604, 0201	Y, N	N/N	L,L	N,N
217	0401 (H)	0301/4 (H)	Y, Y	Y/Y	T,T	Y,Y
218	1501-1502, 0404	0302, 0602	Y, Y	Y/Y	L,L	N,N
219	0404, 1101-1104	0302, 0301/4	Y, Y	Y/Y	L,T	N,Y
220	0101, 1101-1104	0501, 0301/4	N, Y	N/Y	G,T	N,Y
221	1501-1502 (H)	0602 (H)	Y, Y	Y/Y	L,L	N,N
222	0404(H)	0302 (H)	Y, Y	Y/Y	L,L	N,N
223	0701-0702 (H)	0201 (H)	N, N	N/N	L,L	N,N
224	1501-1502, 0701-0702	0201, 0602	N, Y	N/Y	L,L	N,N
225	0401, 1301	0603/8, 0301/4	Y, Y	N/Y	L,T	N,Y
226	1501-1502 (H)	0602 (H)	Y, Y	Y/Y	L,L	N,N
227	0101, 0701-0702	0501, 0303	N, Y	N/Y	G,L	N,N
228	1302, 1101-1104	0604, 0301/4	Y, Y	N/Y	L,T	N,Y
229	0101, 0404	0501, 0302	N, Y	N/Y	G,L	N,N
230	0101, 0401	0501, 0302	N, Y	N/Y	G,L	N,N
231	0404, 1101-1104	0301/4, 0302	Y, Y	Y/Y	T,L	Y,N
232	1501-1502 (H)	0602 (H)	Y, Y	Y/Y	L,L	N,N
233	0101, 0401	0501, 0301/4	N, Y	N/Y	G,T	N,Y
234	0701-0702	0201, 0303	N, Y	N/Y	L,L	N,N
235	0404, 0701-0702	0201, 0302	N, Y	N/Y	L,L	N,N
236	0301, 1303-1304	0201, 0301/4	N, Y	N/Y	L,T	N,Y
237	0701-0702, 1301	0603/8, 0201	Y, N	N/N	L,L	N,N
238	0404, 0301	0201, 0302	N, Y	N/Y	L,L	N,N
239	0701-0702, 1301	0603/8, 0303	Y, Y	N/Y	L,L	N,N

240	0301 (H)	0201 (H)	N, N	N/N	L,L	N,N
241	0401, 1201-1202	0302, 0301/4	Y, Y	Y/Y	L,T	N,Y
242	0801-0805, 1101-1104	0301/4, 0402	Y, N	Y/Y	T,G	Y,N
243	0701-0702, 0801-0805	0201, 0402	N, N	N/Y	L,G	N,N
244	0101, 1301	0501, 0603/8	N, Y	N/N	G,L	N,N
245	1501-1502, 0401	0602, 0302	Y, Y	Y/Y	L,L	N,N
246	0401, 0301	0201, 0301/4	N, Y	N/Y	L,T	N,Y
247	0701-0702, 0404	0201, 0302	N, Y	N/Y	L,L	N,N
248	0701-0702	0603, 0303	Y, Y	N/Y	L,L	N,N
249	0301, 1302	0604, 0201	Y, N	N/N	L,L	N,N
250	1501-1502, 0401	0502, 0301/4	N, Y	N/Y	G,T	N,Y

APPENDIX VIII:

Disease subtype, autoantibody and microsatellite marker allele data for patients from RNHRD, Bath and Hope hospital, Salford.

Patient ID	Disease Subtype	Autoantibodies	Sex	d22s284 1	d22s284 2	d1s419 1	d1s419 2	d2s389 1	d2s389 2	Pdgfb 1	Pdgfb 2	d14s277 1	d14s277 2	d19s400 1	d19s400 2	dxs426 1	dxs426 2
188	dcSSc	RNAP I, II, III	m	106	107	112	113	106	107	108	108	107	108	110	112	105	
137	lcSSc	ACA	f	102	107	104	113	107	107	101	107	107	108	102	106	104	114
179	lcSSc	Positive Unidentified	f	104	109	111	111	105	107	101	101	103	106	112	108	105	106
78	dcSSc	Topo-I	m	102	107	111	114	103	110	101	101	107	107	108	114	105	
251	dcSSc	Negative	f	107	112	111	111	106	114	101	101	105	107	108	116	105	105
26	lcSSc	ACA	f	102	108	106	110	103	107	101	101	107	108	108	108	105	114
224	dcSSc	Ro, La	m	102	107	106	110	107	112	101	101	107	108	108	112	105	
25	lcSSc	ACA	f	107	109	111	111	107	112	101	107	105	108	110	110	114	114
270	dcSSc	RNAP I, II, III	m	107	107	109	115	107	111	105	107	105	105	106	118	105	
27	lcSSc	ACA	f	108	109	107	112	107	112	101	101	106	107	102	108	105	105
256	lcSSc	Ro, La, U3RNP	f	105	107	110	112	107	107	101	101	107	107	102	108	104	105
66	dcSSc	RNAP I, II, III	f	105	106	109	110	107	107	101	101	108	109	108	108	105	105
280	lcSSc	Negative	f	106	107	111	113	106	107	101	101	103	106	102	118	106	114
272	dcSSc	RNAP, I, II wk, III	f	106	107	110	112	112	113	101	105	107	109	104	114	105	105
43	dcSSc	RNAP I, II, III	f	108	102	106	114	106	107	101	101	107	107	108	110	105	115
4	lcSSc	Wk. Topo-I	m	102	107	114	116	111	112	101	108	103	105	108	114	105	105
36	dcSSc	RNAP I, II, III	f	106	112	111	114	105	107	101	101	108	109	104	108	105	105
209	dcSSc	Topo-I	m	107	107	106	112	103	107	101	101	107	109	108	110	105	
263	lcSSc	Negative	f	106	108	106	111	107	111	101	101	105	108	102	110	105	105
264	lcSSc	ACA	f	108	108	112	114	111	114	101	108	107	107	104	108	105	106
269	lcSSc	Wk U1RNP	f	102	107	111	111	110	115	101	101	106	107	112	112	105	105
74	lcSSc	ACA	f	107	107	111	113	112	115	101	106	107	108	108	112	104	105
206	lcSSc	ACA	f	106	107	108	114	107	113	101	101	102	106	108	112	105	114

267	lcSSc	Positive Unidentified	f	105	107	104	106	106	107	101	107	107	108	102	108	105	114
46	lcSSc	Positive Unidentified	f	108	109	108	113	106	116	101	101	107	108	110	114	106	106
228	lcSSc	U1RNP	m	106	107	106	108	107	112	101	101	105	107	108	116	105	106
107	lcSSc	Negative	f	108	108	112	113	107	107	101	101	107	108	110	116	106	114
212	lcSSc	Pm-Scl	f	106	108	106	113	106	107	102	108	106	106	106	114	105	105
146	lcSSc	ACA	f	102	106	106	111	107	112	101	101	103	106	110	114	106	114
71	lcSSc	ACA	f	108	108	111	111	106	107	107	107	107	108	102	104	105	105
247	lcSSc	Failed Sample (Hint of Pm-Scl but not confirmed)	f	102	107	110	115	101	114	101	101	103	106	112	114	105	106
28	dcSSc	Multiple unidentified bands	m	102	105	106	108	107	111	101	107	105	107	108	116	105	
240	lcSSc	ACA Wk.	f	102	112	111	111	106	114	101	108	107	107	104	112	105	105
31	lcSSc	ACA	m	108	109	111	111	107	114	101	101			108	108	105	
69	lcSSc	ACA	f	107	108	111	112	107	107	101	108	106	106	110	112	105	114
253	lcSSc	ACA	f	109	109	106	107	107	107	103	108	106	107	102	116	104	106
244	dcSSc	ACA	f	106	108	111	111	103	115	101	101	107	108	102	108	103	105
21	lcSSc	ACA	m	105	107	107	111	107	107	101	101	102	107	110	114	115	
34	dcSSc	RNAP I, II, III	f	105	107	110	111	111	115	102	102	103	107	102	112	105	116
167	lcSSc	ACA	f	104	107	111	111	107	107	101	108	105	107	108	116	105	114
177	dcSSc	ACA	f	106	108	108	111	103	115	107	101	103	107	114	114	105	105
70	lcSSc	RNAP II, Topo-I	f	104	109	107	114	107	107	107	108	103	120	102	106	105	114
86	lcSSc	Weak Unidentified	m	108	108	106	108	106	111	101	101	107	107	110	110	114	
175	dcSSc	RNAP I, IIwk, III	f	107	109	104	105	111	116	108	108	102	107	112	114	105	116
149	lcSSc	Negative	f	104	107	109	110	106	113	107	107	106	111	102	112	105	105
230	lcSSc	ACA	f	106	110	108	115	112	114	101	101	107	107	108	114	104	105
181	lcSSc	ACA	m	101	106	112	113	103	107	101	108	103	110	112	114		
6	dcSSc	La	f	106	107	106	115	106	111	107	107	107	107	108	108	105	115
210	lcSSc	Ro, La	f	106	108	106	106	103	111	101	101	107	120	114	114	106	114
85	dcSSc	Topo-I	f	106	107	107	111	107	107	101	106	107	109	110	116	105	105
61	lcSSc	ACA	f	107	107	106	110	107	115	101	101	103	107	102	114	105	106
255	lcSSc	U1RNP	f	107	107	110	111	106	112	107	106	106	107	102	102	105	116
187	lcSSc	ACA	f	106	107	106	106	112	115	101	101	107	107	108	112	104	108

144	lcSSc	Pm-Scl	f	107	108	106	107	106	115	101	101	115	117	102	114	105	105
72	lcSSc	ACA	f	102	112	106	111	106	114	101	107	106	107	114	114	105	105
22	lcSSc	Jo-I	f	104	106	111	111	107	119	108	108	102		102	112	106	117
18	lcSSc	Negative	f	102	106	106	111	109	112	101	107	103	106	108	114	105	105
79	lcSSc	ACA	f	102	107	110	112	106	111	101	101	106	108	106	108	105	105
52	lcSSc	Positive Unidentified	f	107	109	106	116	107	111	101	101	106	107	114	114	108	115
213	dcSSc	Topo-I	m	102	105	111	113	103	115	101	107	107	107	108	114	104	
180	lcSSc	ACA	f	103	107	106	112	113	115	101	101	107	107	108	108	105	115
53	lcSSc		f	105	106	106	106	106	112	101	107	106	108	114	116	105	114
95	dcSSc	Negative	f	104	107	106	112	106	112	108	108	107	107	110	118	114	115
87	lcSSc	Pm-Scl	f	104	107	106	113	112	115	101	108	106	106	102	110	105	105
89	lcSSc	ACA	f	102	105	107	113	107	107	101	101	103	106	106	114	104	104
202	lcSSc	Failed Sample (Hint of Pm-Scl but not confirmed)	f	102	110	109	113	111	115	101	101	106	107	108	114	105	115
162	lcSSc	Negative	f	102	108	111	114	103	114	101	101	105	107	108	114	105	114
54	dcSSc		f	104	106	111	114	103	107	101	101	108	110	112	116	105	115
55	lcSSc		f	107	109	105	111	107	107	107	107	102	107	102	116	131	113
205	lcSSc	Positive Unidentified	f	106	107	102	107	106	107	101	101	106	106	106	108	105	105
17	SSc	Positive Unidentified	f	106	107	107	112	106	112	101	101	106	107	108	112	105	105
132	lcSSc	RNAP I, II, III	f	106	107	113	118	103	114	101	101	106	107	110	112	105	115
139	lcSSc	Pm-Scl	f	107	109	108	111	107	115	101	101	107	107	108	110	105	105
56	lcSSc		f	104	107	111	119	107	115	101	101	106	107	104	106	105	105
118	dcSSc	Topo-I	f	102	107	106	114	106	112	101	101	102	108	108	110	105	106
133	lcSSc	Ro, Pm-Scl	f	107	108	107	112	107	107	101	101	103	104	108	108	105	106
268	lcSSc	ACA	f	105	107	107	111	107	113	101	106	107	107	108	112	105	114
222	lcSSc	ACA	f	102	107	104	111	107	111	108	101	103	108	108	116	105	108
56	lcSSc		f	102	107	104	111	106	114	101	101	107	107	102	114	106	114
229	lcSSc	ACA	f	104	107	104	113	105	107	101	107	106	106	110	112	105	106
140	lcSSc	ACA	f	107	107	107	111	107	115	101	101	107	114			106	106
136	lcSSc	ACA	f	104	106	111	113	107	113	101	107	102	107			105	105
129	dcSSc	RNAP I, II wk, III	f	106	107	106	111	103	107	101	101	107	107	108	114	104	105

110	dcSSc	Negative	m	102	107	111	112	114	115	101	102	102	109	108	108	115	115
211	dcSSc	Positive Unidentified	f	102	108	113	113	114	115	101	101	103	105	106	114	105	105
97	lcSSc	Negative	f	102	107	106	113	107	112	102	107	107	107	108	114	106	114
112	dcSSc	RNAP I, II, III	f	101	107	107	110	115	115	101	103	104	107	108	112	105	105
143	dcSSc	RNAP I, III	f	102	106	112	113	106	107	101	101	105	107	102	114	105	113
141	lcSSc	Positive Unidentified	f	102	106	113	116	107	116	101	102	102	108	102	108	X	X
57	lcSSc		f	102	107	109	114	107	112	101	101	107	107	108	110	104	114
199	lcSSc	U1RNP	f	106	107	102	111	105	107	101	101	107	108	102	114	105	105
135	lcSSc	Positive Unidentified	f	105	106	104	109	107	107	101	101	107	108	108	116	113	114
138	lcSSc	ACA	f	106	108	111	111	103	106	107	108	107	107	110	112	105	106
58	lcSSc		f	105	107	110	110	107	114	102	107	108	109	110	112	105	105
246	dcSSc	La	f	102	104	108	110	106	107	101	107	107	107	108	112	105	105
231	lcSSc	ACA	f	107	109	102	111	103	106	101	107	102	109	106	110	105	105
145	dcSSc	Topo-I	f	102	107	107	114	106	116	102	107	103	107	104	114	104	105
106	lcSSc	Jo-I	f	106	107	106	114	107	111	101	101	102	107	112	116	105	105
249	lcSSc	U1RNP	f	107	107	107	109	107	112	101	101	102	107	108	112	114	116
241	lcSSc	Positive Unidentified	f	102	107	108	112	106	110	101	101	106	107	104	114	105	105
186	lcSSc	Ro, La	f	106	108	111	113	103	115	101	101	107	107	108	110	105	105
274	dcSSc	Topo-I	f	104	106	111	112	103	107	101	107	106	108			107	115
190	dcSSc	Topo-I	f	106	107	108	111	107	106	107	107	107	108	108	108	105	105
183	lcSSc	Negative	f	102	107	106	107	106	107	102	108	107	107	108	110	105	109
221	lcSSc	ACA & Positive unidentified	f	106	107	111	113	107	111	101	103	106	107	102	112	105	114
197	lcSSc	ACA	f	104	107	111	113	107	113	103	107	107	107	110	116	105	116
200	lcSSc	Negative	f	103	108	114	114	103	107	102	108	107	107	108	114	105	114
217	lcSSc	RNAP I, III	f	106	107	107	111	106	115	101	101	105	120	108	114	105	118
232	lcSSc	Pm-Scl	m	104	107	102	107	107	107	101	101	102	108	114	118	106	106
203	lcSSc	Pm-Scl	f	102	109	106	111	106	115	102	102	106	108	108	110	106	114
196	lcSSc	ACA	f	102	108	106	109	103	106	101	102	103	106	108	116	105	105
195	lcSSc	ACA	f	107	107	102	112	106	112	102	107	106	106	102	102	x	x
227	lcSSc	Positive Unidentified	m	107	107	106	108	115	115	101	120	103	107	112	112	105	105
218	dcSSc	Topo-I	f	102	107	106	113	111	114	101	103	105	106	110	112	104	111

192	lcSSc	U1RNP	m	107	108	106	111	107	112	101	101	107	109	108	114	105	105
194	lcSSc	ACA	f	107	108	110	110	111	112	101	105	107	108	114	116	105	114
193	lcSSc	ACA Wk.	f	102	106	109	113	106	107	101	102	106	107	102	102	105	105
233	lcSSc	Negative	f	104	107	111	112	107	115	101	107	108	110	106	116	106	106
257	lcSSc	ACA Wk.	m	104	107	X	X	107	115	101	101	107	107	110	110	105	105
191	lcSSc	ACA	f	104	108	104	111	107	106	101	101	103	106	110	110	105	105
219	lcSSc	Negative	m	106	107	106	113	107	112	102	107	106	108	102	108	108	108
265	lcSSc	ACA	f	109	109	102	107	107	107	102	108	107	108	108	114	106	111
216	dcSSc	V. Wk. Topo-I	m	104	109	111	113	107	111	101	109	102	107	108	108	105	105
245	lcSSc	Jo-I	m	106	107	111	115	106	111	101	107	105	106	108	108	105	105
59	dcSSc		m	107	112	106	111	107	113	101	102	107	108	112	112	108	108
60	lcSSc		f	104	107	111	114	107	107	101	108	104	105	106	110	104	105
239	lcSSc	ACA	f	102	107	108	113	107	115	101	101	102	106	102	108	105	116
220	lcSSc		m	107	109	108	116	106	112	101	101	107	107	108	112	104	104
248/261	dcSSc	Negative	m	102	108	111	119	106	106	107	107	106	107	103	108	106	106
266	lcSSc	ACA	f	108	109	107	110	114	115	102	102	106	107	108	112	105	114
262	lcSSc	U1RNP	f	105	108	102	106	103	107	101	101	107	108	104	112	105	114
61	dcSSc		f	106	107	111	113	103	106	102	108	107	107	106	108	105	105
260	lcSSc	ACA	f	107	108	111	111	105	106	101	107	106	107	102	110	105	105
204	lcSSc	ACA	f	106	107	107	111	107	112	101	107	106	107	112	114	105	115
62	lcSSc		f	102	107	106	111	107	115	107	108	106	107	108	110	105	106
63	lcSSc		f	104	106	111	114	106	107	101	101	105	108	114	116	104	114
64	lcSSc		f	107	106	112	112	107	107	101	106	107	108	103	108	105	114
65	lcSSc		f	102	106	116	116	114	115	101	103	102	106	108	114	105	105
66	lcSSc		f	102	106	107	111	106	107	107	109	107	107	108	110	106	114
223	lcSSc		f	106	107	111	111	106	112	101	101	106	107	101	114	105	116
(Bath	Patients)																
1	SSc	ACA	f	106	107	104	106	X	X	X	X	107	109	104	108	X	X
2	SSc	ACA/ AMA	f	102	107	106	111	107	115	101	107	102	107	106	110	105	105
3	SSc	Pm-Scl	f	102	107	106	111	107	115	X	X	107	108	102	116	105	106
4	SSc	ACA	f	105	106	104	111	105	107	101	101	102	106	102	114	105	114

5	SSc	RNAP I, IIO, IIA, III	f	106	107	111	112	106	113	101	106	106	107	108	116	105	114
6	SSc	ACA	f	107	108	109	111	106	107	101	101	107	108	112	116	105	105
7	SSc	ACA	f	106	109	111	112	113	115	102	107	107	107	102	114	105	114
8	SSc	Topo-I	f	102	107	111	113	107	107	101	103	107	107	108	114	104	105
9	SSc	ACA	f	102	107	111	115	111	113	101	101	107	108	110	112	105	106
10	SSc	Topo-I	f	106	108	105	115	106	115	102	107	108	108	108	114	X	X
11	SSc	ACA	f	106	107	106	111	107	107	X	X	106	108	102	102	X	X
12	SSc	ACA	f	104	107	111	114	106	113	101	101	107	107	104	110	105	115
13	SSc	ACA	f	106	107	107	112	111	115	101	101	104	106	114	114	105	106
14	SSc	ACA	f	102	109	111	111	103	111	101	107	107	108	110	114	104	115
15	SSc	Pos. Unid.	f	107	107	106	108	106	115	101	108	107	107	108	110	104	114
16	SSc	DCNS, ENA Neg.	m	102	107	109	116	106	112	101	101	107	108	102	108	105	
17	SSc	ACA	f	107	107	108	111	106	106	101	101	102	107	108	116	105	106
18	SSc	Jo-I	f	109	110	111	115	106	115	103	107	107	107	108	108	105	105
19	SSc	ACA	f	102	107	106	112	107	107	101	101	108	108	108	108	104	115
20	SSc	ACA	f	107	108	107	110	107	107	101	101	107	108	108	110	105	106
21	SSc	ACA	f	102	106	107	114	106	115	101	103	107	107	108	114	104	105
22	SSc	ACA	f	102	107	106	113	107	107	101	101	107	108	110	112	114	114
23	SSc	ThRNP	f	106	107	106	112	106	115	101	101	102	107	114	116	114	115
24	SSc	ACA	f	102	107	106	111	X	X	101	101	106	107	102	114	105	114
25	SSc	Jo-I	f	105	106	110	111	101	114	101	101	107	108	102	110	105	105
26	SSc	La, Pm-Scl	f	102	109	106	106	106	106	101	107	107	107	110	116	105	114
27	SSc	Topo-I, RNAP IIO, IIA	m	102	106	110	111	107	112	101	101	107	109	110	112	130	
28	SSc	ACA	f	102	104	102	112	103	112	101	101	102	108	112	116	105	109
29	SSc	ACA	f	108	109	107	111	106	115	101	107	106	108	108	x	105	105
30	SSc	ACA	f	102	111	111	112	103	112	101	101	105	107	104	108	105	114
31	SSc	ACA	f	108	110	111	116	103	107	101	101	102	106	102	114	105	114
32	SSc	ACA	f	102	102	107	110	109	112	101	102	106	107	102	106	105	105
33	SSc	Topo-I, Ro, Jo-I	f	107	107	110	113	106	108	101	101	107	108	102	110	105	114
34	SSc	Scl-70 by ENA	f	108	109	110	115	107	115	107	108	107	108	114	116	104	114

35	SSc	ACA	f	106	108	102	112	107	111	101	107	105	108	110	110	105	109
36	SSc	ACA	m	102	109	111	112	112	113	101	101	107	107	110	114	108	
37	SSc	Topo-I, RNAP IIO	f	106	107	106	111	107	112	101	106	107	107	108	108	105	114
38	SSc	ACA	f	101	107	107	111	106	111	101	101	106	109	102	116	104	105
39	SSc	Topo-I, RNAP IIO	f	107	108	111	113	106	107	101	103	102	108	110	112	106	107
40	SSc	Ro, La	f	106	107	112	115	103	106	101	101	107	108	114	114	105	114
41	SSc	ACA	f	101	107	111	111	112	113	X	X	102	108	114	116	114	115
42	SSc	RNAP I, IIO, IIA, III	f	105	107	X	X	112	115	101	101	104	107	110	110	X	X
43	SSc	Ro	m	108	108	108	110	106	106	101	101	107	108	108	118	105	
44	SSc	ACA	f	107	108	107	115	110	111	X	X	107	108	112	115	105	105
45	SSc	Pos. Unid.	f	107	107	107	111	X	X	103	107	106	108	102	116	X	X
46	SSc	Ro, La, U3RNP	m	107	108	107	115	106	115	101	103	106	107	106	108	105	
47	SSc	Topo-I, U1RNP+/-Sm	f	106	107	103	110	103	114	101	106	106	107	110	112	105	106
48	SSc	ACA	f	107	109	108	111	106	112	101	101	107	108	108	110	105	114
49	SSc	Various ANA	f	107	107	105	112	X	X	X	X	107	107	108	114	X	X
50	SSc	Topo-I, RNAP IIO	f	102	107	106	112	106	111	101	106	107	108	108	112	105	116
51	SSc	RNAP I, III	f	102	107	107	111	107	107	101	101	106	108	102	104	104	114

APPENDIX IX:

Publications

Susol E, **Rands AL**, Herrick A, McHugh N, Barrett JH, Ollier WE, Worthington J. Association of markers for TGFb3, TGFb2 and TIMP1 with systemic sclerosis. Rheumatology, 2000: 39, 1332-1336

A.L.Rands, G.R.Harvey, E.Susol, A.Herrick, N.J.McHugh. Immunogenetics and clinical features in scleroderma patients with either anti-topoisomerase-I or anti-RNA polymerase III antibodies. The 6th International workshop for scleroderma research. 2000. Abstract. Awarded: ***The symposium prize*** for outstanding work.

AL Rands, J Whyte, B Cox, ND Hall, NJ McHugh. MHC class II associations with autoantibody and T cell immune responses to the scleroderma autoantigen topoisomerase-I. Journal of Autoimmunity. 2000: 15, 451-458.

J.Dunphy, M. Oliver, **A.L.Rands**, C.R.Lovell and N.J.McHugh. Antineutrophil cytoplasmic antibodies and HLA class II alleles in minocycline-induced lupus-like syndrome. British Journal of Dermatology. 2000: 142 (3): 461-468.

E Susol, **AL Rands**, A Herrick, N McHugh, JH Barrett, WER Ollier, J Worthington. Association of markers for TGFβ2, TGFβ3 and TIMP1 with systemic sclerosis. Rheumatology, 2000; 39:139

Harvey, G.R., Butts, S., **Rands, A.L.**, Patel, Y. McHugh, N.J. Clinical and serological associations with anti-RNA polymerase antibodies in systemic sclerosis. Clinical and Experimental Immunology. 1999: 117: 395-402.

A. L. Rands, A. Kumar, J. Dunphy, P. Owen, B. Cox, L. Daly, N. J. McHugh. Clinical features, immunogenetics and outcome in individuals with anti-centromere antibodies. Annals of the Rheumatic Diseases. XIV European League Against Rheumatism Congress. 1999. Abstract.

J. Dunphy, **A Rands**, B. Cox, N. J. McHugh. ANCA positivity and HLA Class II antigen in minocycline-associated lupus-like illness. Annals of the Rheumatic Diseases. XIV European League Against Rheumatism Congress. 1999. Abstract.

P.A.Owen, B. Cox, **A. Rands**, A. Al-Saidi, N.J.McHugh. The serological profile of autoantibody reactivity to the U1snRNP particle and its relationship to HLA DRB1 and DQB1 alleles. Annals of the Rheumatic Diseases. XIV European League Against Rheumatism Congress. 1999. Abstract.

Alison Rands, Jean Whyte, Nicolas Hall, Neil McHugh. MHC Class II associations with B and T cell immune responses to topoisomerase-I. British Society for Rheumatology XIVth AGM. 1997. Abstract.

Juliet Dunphy, **Alison Rands**, and Neil J McHugh. Anti-myeloperoxidase antibodies in minocycline lupus syndrome. British Society for Rheumatology XIVth AGM. 1997. Abstract.

GR Harvey, **A Rands**, PJ Maddison, NJ McHugh. Antibodies to RNA polymerases in systemic sclerosis: clinical and serological associations. British Society for Rheumatology XIVth AGM 1996 Abstract.

GR Harvey, **A Rands**, NJ McHugh. Anti-RNA polymerase antibodies in systemic sclerosis: association with anti-topoisomerase I antibodies and identification of autoreactive subunits of RNA polymerase II. Clinical and Experimental Immunology. 1996, **105** (3): 468-474.

Georgina R. Harvey, **Alison Rands**, Yusuf I. Patel, Peter J. Maddison, and Neil J. McHugh. Antibodies to RNA Polymerases in Systemic Sclerosis: Clinical and Serological Associations. American College of Rheumatology. 60th AGM. 1996. Abstract.

Neil J. McHugh, Juliet Dunphy, **Alison Rands**, and Colin Hill.
Anti-Myeloperoxidase Antibodies in Minocycline-Induced Lupus Syndrome And Myeloperoxidase Dependent Drug Cytotoxicity. American College of Rheumatology. 60th Annual General Meeting. 1996. Abstract.

BIBLIOGRAPHY

BIBLIOGRAPHY

(1) Perez MI, Kohn SR. Systemic sclerosis. J Am Acad Dermatol 1993; 28:525-547.

(2) LeRoy EC, Black C, Fleischmajer R, Jablonska S, Krieg T, Medsger TA, Jr. et al. Scleroderma (systemic sclerosis): classification, subsets and pathogenesis. J Rheumatol 1988; 15:202-204.

(3) Silver RM. Clinical problems: The lungs. Rheum Dis Clin North Am 1996; 22:825-840.

(4) Fagundus DM, LeRoy EC. Systemic sclerosis (scleroderma). In: Belch JJF, Zurier RB, editors. Connectice tissue diseases. London: Chapman & Hall, 1995: 51-64.

(5) Silver RM, Miller KS. Lung involvement in systemic sclerosis. Rheum Dis Clin North Am 1990; 16:199-216.

(6) Steen VD. Scleroderma renal crisis. Rheum Dis Clin North Am 1996; 22:861-878.

(7) Medsger TA, Jr. Systemic sclerosis (scleroderma), Localized scleroderma, eosinophilic fasciitis, and calcinosis. In: McCarty DJ, editor. Arthritis and allied conditions. A textbook of rheumatology. Philadelphia. London.: Lea & Febiger, 1989: 1119-1165.

(8) Young MA, Rose S, Reynolds JC. Gastrointestinal manifestations of scleroderma. Rheum Dis Clin North Am 1996; 22:797-823.

(9) Black CM, Stephens C. Systemic sclerosis (scleroderma) and related disorders. Oxford textbook of rhuematology. Oxford University Press, 1993: 771-789.

(10) Cerinic MM, Generini S, Pignone A, Casale R. The nervous system in systemic sclerosis (scleroderma): Clinical features and pathogenetic mechanisms. Rheum Dis Clin North Am 1996; 22:879-892.

(11) Wigley FM. Clinical aspects of systemic and localized scleroderma. Curr Opin Rheumatol 1994; 6:628-636.

(12) Poormoghim H, Lucas M, Fertig N, Medsger TA, Jr. Systemic sclerosis sine scleroderma: Demographic, clinical, and serologic features and survival in forty-eight patients. Arthritis Rheum 2000; 43:444-451.

(13) Venables PJW. Miscellaneous Disorders: Overlap syndromes. In: Klippel JH, Dieppe PA, editors. Rheumatology. London: Mosby International, 1998: 28.1-28.7.

(14) Jablonska S, Blaszczyk M. Scleroderma overlap syndromes. Adv Exp Med Biol 1999; 455:85-92.

(15) Medsger TA, Jr., Silman AJ, Steen VD, Black CM, Akesson A, Bacon PA et al. A disease severity scale for systemic sclerosis: Development and testing. J Rheumatol 1999; 26:2159-2167.

(16) Barnett AJ. Scleroderma (progressive systemic sclerosis): progress and course based on a personal series of 118 cases. Med J Aust 1978; 12:129-134.

(17) Giordano M, Valentini G, Migliaresi S, Picillo U, Vatti M. Different antibody patterns and different prognoses in patients with scleroderma with various extent of skin sclerosis. J Rheumatol 1986; 113:911-916.

(18) Silman AJ. Scleroderma - Demographics and survival. J Rheumatol 1997; 24:58-61.

(19) Silman AJ. Scleroderma and survival. Ann Rheum Dis 1991; 50:267-269.

(20) Mayes M. Scleroderma Epidemiology. Rheum Dis Clin North Am 1996; 22:751-764.

(21) Laing TJ, Gillespie BW, Toth MB, Mayes MD, Gallavan RHJr, Burns CJ et al. Racial differences in scleroderma among women in Michigan. Arthritis Rheum 1997; 40:734-742.

(22) Steen VD, Medsger TA, Jr. Epidemiology and natural history of systemic sclerosis. Rheum Dis Clin North Am 1990; 16:1-9.

(23) Reveille JD, Durban E, Goldstein R, Moreda R, Arnett FC. Racial differences in the freqencies of scleroderma-related autoantibodies. Arthritis Rheum 1992; 35:216-218.

(24) Kuwana M, Okano Y, Kaburaki J, Tojo T, Medsger TA, Jr. Racial differences in the distribution of systemic sclerosis-related serum antinuclear antibodies. Arthritis Rheum 1994; 37:902-906.

(25) Arnett F. HLA and autoimmunity in scleroderma (systemic sclerosis). Int Rev Immunol 1995; 12:107-128.

(26) Briggs D, Welsh KI. Major histocompatibility complex class II genes and systemic sclerosis. Ann Rheum Dis 1991; 50 Suppl. 4:862-865.

(27) Mayes MD. Classification and epidemiology of scleroderma. Seminars in Cutan Med Surg 1998; 17:22-26.

(28) Silman AJ, Howard Y, Hicklin AJ, Black C. Geographical clustering of scleroderma in south and west London. Brit J Rheumatol 1990; 29:92-96.

(29) Silman AJ, Jannini S, Synnons D, Bacon P. An epidemiological study of scleroderma in the West Midlands. Brit.J.Rheumatol. 27, 286-290. 1988.

(30) Harley JB, Neas BR. Oklahoma Choctaw and systemic sclerosis: The Founder effect and genetic susceptibility. Arthritis Rheum 1998; 41:1725-1728.

(31) Arnett FC, Howard RF, Tan F, Moulds JM, Bias WB, Durban E et al. Increased prevalence of systemic sclerosis in a native American tribe in Oklahoma. Association with an Amerindiam HLA haplotype. Arthritis Rheum 1996; 39:1362-1370.

(32) Campbell PM, LeRoy EC. Pathogenesis of systemic sclerosis: a vascular hypothesis. Semin Arthritis Rheum 1975; 4:351-368.

(33) Hunt BJ, Jurd KM. Endothelial cell activation: A central pathophysiological process. BMJ 1998; 316:1328-1329.

(34) Smith EA, LeRoy EC. Systemic sclerosis: Etiology and pathogenesis. In: Klippel JH, Dieppe PA, editors. Rheumatology. London: Mosby International, 1998: 9.1-9.10.

(35) Furst DE, Clements PJ. Hypothesis for the pathogenesis of systemic sclerosis. J Rheumatol 1997; 24:53-57.

(36) Seibold JR. Systemic sclerosis: Clinical features. In: Klippel JH, Dieppe PA, editors. Rheumatology. London: Mosby International, 1998: 8.1-8.14.

(37) White B. Immunopathogenesis of systemic sclerosis. Rheum Dis Clin North Am 1996; 22:695-708.

(38) LeRoy EC. A brief overview of the pathogenesis of scleroderma (systemic sclerosis). Ann Rheum Dis 1992; 51:286-288.

(39) Varga J, Rudnicka L, Vitto J. Connective tissue alterations in systemic sclerosis (SSc). Clin Dermatol 1994; 12:387-396.

(40) LeRoy EC, Smith EA, Kahaleh MB, Trojanowska M, Silver RM. A strategy for determining the pathogenesis of systemic sclerosis. Is transforming growth factor β the answer? Arthritis Rheum 1989; 7:817-825.

(41) Postlethwaite AE. Connective tissue metabolism including cytokines in scleroderma. Curr Opin Rheumatol 1993; 5:766-772.

(42) ota H, Kumagai S, morinobu A, Nakao K. Enhanced production of transforming growth factor-beta (TGF-β) during autologous mixed lymphocyte reaction in SSc patients. Clin Exp Immunol 1995; 100:99-103.

(43) Casciola-Rosen L, Wigley F, Rosen A. Scleroderma autoantigens are uniquely fragmented by metal-catalyzed oxidation reactions: Implications for pathogenesis. J Exp Med 1997; 185:71-79.

(44) Rosen A, Casciola-Rosen L, Wigley F. Role of metal-catalyzed oxidation reactions in the early pathogenesis of scleroderma. Curr Opin Rheumatol 1997; 9:538-543.

(45) Harvey GR, McHugh NJ. Serological abnormalities in systemic sclerosis. Curr Opin Rheumatol 1999; 11:495-502.

(46) Kuwana M, Medsger TA, Jr., Wright TM. T and B cell collaboration is essential for the autoantibody response to DNA topoisomerase I in systemic sclerosis. J Immunol 1995; 155:2703-2714.

(47) Sarkozi J, Bookman AAM, Lee P, Keystone EC, Fritzler MJ. Significance of anticentromere antibody in idiopathic Raynaud's syndrome. Am J Med 1987; 83:893-898.

(48) Youinou P, Revelen R, Bordron A. Is antiendothelial cell antibody the murder weapon in systemic sclerosis? Clinical & Experimental Rheumatology 1999; 17:35-36.

(49) Carvalho D, Savage COS, Black CM, Pearson JD. IgG antiendothelial cell autoantibodies from scleroderma patients induce leukocyte adhesion to human vascular endothelial cells in vitro. J Clin Invest 1996; 97:111-119.

(50) Osborne BA. Apoptosis and the maintenance of homoeostasis in the immune system. Curr Opin Immunol 1996; 8:245-254.

(51) Bordron A, Dueymes M, Levy Y, Jamin C, LeRoy J-C, Shoenfeld Y et al. The binding of some human antiendothelial cell antibodies induces endothelial cell apoptosis. J Clin Invest 1998; 101:2029-2035.

(52) Sgonc R. The vascular perspective of systemic sclerosis: of Chickens, mice and men. Int Arch Allergy Immunol 1999; 120:169-176.

(53) Okano Y. Antinuclear antibody in systemic sclerosis (scleroderma). Rheum Dis Clin North Am 1996; 22:709-735.

(54) Hsieh T. DNA topoisomerases. Curr Opin Cell Biol 1992; 4:396-400.

(55) Moroi Y, Peebles C, Fritzler MJ, Steigerwald J, Tan EM. Autoantibody to centromere (kinetochore) in scleroderma sera. Proc Natl Acad Sci USA 1980; 77:1627-1631.

(56) Earnshaw WC, Tomkiel JE. Centromere and kinetochore structure. Curr Opin Cell Biol 1992; 4:86-93.

(57) McHugh.N.J. Centromere Autoantibodies. In: Peter.J.B., Shoenfeld.Y, editors. Autoantibodies. Elsevier Science B.V., 1996: 161-167.

(58) Steen VD, Ziegler GL, Rodnan GP, Medsger TA, Jr. Clinical and laboratory associations of anticentromere antibody in patients with progressive systemic sclerosis. Arthritis Rheum 1984; 27:125-131.

(59) Pollard KM, Reimer G, Tan EM. Autoantibodies in scleroderma. Clin Exp Rheumatol 1989; 7/S-3:57-62.

(60) Wigley FM, Wise RA, Miller R, Needleman BW, Spence RJ. Anticentromere antibody as a predictor of digital ischemic loss in patients with systemic sclerosis. Arthritis Rheum 1992; 35:688-693.

(61) Oddis CV, Okano Y, Rudert WA, Trucco M, Duquesnoy RJ, Medsger TA, Jr. Serum autoantibody to the nucleolar antigen PM-Scl: Clinical and immunogenetic associations. Arthritis Rheum 1992; 35:1211-1217.

(62) Okano Y, Medsger TA, Jr. Autoantibody to Th ribonucleoprotein (nucleolar 7-2 RNA protein particle) in patients with systemic sclerosis. Arthritis Rheum 1990; 33:1822-1828.

(63) Steen VD, Powell VD, Medsger TA, Jr. Clinical correlations and prognosis based on serum autoantibodies in patients with systemic sclerosis. Arthritis Rheum 1988; 31:196-203.

(64) Fanning GC, Welsh KI, Bunn C, Du Bois R, Black CM. HLA associations in three mutually exclusive autoantibody subgroups in UK systemic sclerosis patients. Brit J Rheumatol 1998; 37:201-207.

(65) Nelson CA, Fremont DH. Structural principles of MHC class II antigen presentation. Reviews in immunogenetics 1999; 1:47-59.

(66) McCluskey J. The human leukocyte antigens and clinical medicine: an overview. Reviews in immunogenetics 1999; 1:3-20.

(67) Garca KM, Chan VSF, Ohashi PS. T Cell tolerance and autoimmunity. Reviews in immunogenetics 2000; 2:2-17.

(68) Schiffenbauer J, Schwartz BD. The HLA comlex and its relationship to rheumatic diseases. Rheum Dis Clin North Am 1987; 13:463-485.

(69) Wicker LS. Major Histocompatibility Complex-linked control of autoimmunity. J Exp Med 1997; 186:973-975.

(70) Nepom GT, Erlich H. MHC class-II molecules and autoimmunity. Annu Rev Immunol 1991; 9:493-525.

(71) Steinman L. Escape from "Horror Autotoxicus": pathogenesis and treatment of autoimmune disease. Cell 1995; 80:7-10.

(72) Pisetsky DS. Rheumatic disease etiology: Immune-mediated inflammation. In: Klippel JH, Dieppe PA, editors. Rheumatology. London: Mosby International, 1998: 13.1-13.6.

(73) Gregersen PK, Silver J, Winchester RJ. The shared epitope hypothesis. An approach to understanding the molecular genetics of susceptibility to rheumatoid arthritis. Arthritis Rheum 1987; 30:1205-1213.

(74) Todd JA, Acha-Orbea H, Bell JI, Chao N, Fronek Z, Jacob CO et al. A molecular basis for MHC Class II - associated autoimmunity. Science 1988; 240:1003-1009.

(75) Das P, Abraham R, David C. HLA transgenic mice as models of human autoimmune diseases. Reviews in immunogenetics 2[1], 105-114. 2000.

(76) Seglias J, Li EK, Cohen MG, Wong RWS, Potter PK, So AK. Linkage between rheumatoid arthritis susceptibility and the presence of HLA-DR4 and DRβ allelic third hypervariable region seqences in Southern Chinese persons. Arthritis Rheum 1992; 35:163-167.

(77) Nepom GT, Byers P, Seyfried C, Healey LA, Wilske K, Stage D et al. HLA genes associated with rheumatoid arthritis. Identification of susceptibility alleles using specific oligonucleotide probes. Arthritis Rheum 1989; 32:15-21.

(78) Reveille JD. Molecular genetics of systemic sclerosis. Curr Opin Rheumatol 1995; 7:522-528.

(79) Gladman DD, Keystone EC, Baron M, Lee P, Cane D, Mervert H. Increased frequency of HLA-DR5 in scleroderma. Arthritis Rheum 1981; 24:854-856.

(80) Genth E, Mierau R, Genetzky P, von Muhlen CA, Kaufmann S, von Wilmowsky H et al. Immunogenetic associations of scleroderma-related antinuclear antibodies. Arthritis Rheum 1990; 33:657-665.

(81) Morel PA, Chang HJ, Wilson JW, Conte C, Falkner D, Tweardy DJ et al. HLA and ethnic associations among systemic sclerosis patients with anticentromere antibodies. Hum Immunol 1995; 42:35-42.

(82) Vlachoyiannopoulos PG, Dafni UG, Pakas I, Spyropoulou-Vlachou M, Stavropoulos-Giokas C, Moutsopoulos HM. Systemic scleroderma in Greece: low mortality and strong linkage with HLA-DRB1*1104 allele. Ann Rheum Dis 2000; 59:359-367.

(83) Vargas-Alarcon G, Granados J, Ibanez De Kasep G, Alcocer-Varela J, Alarcon-Segovia D. Association of HLA-DR5 (DR11) with systemic sclerosis (scleroderma) in Mexican patients. Clinical & Experimental Rheumatology 1995; 13:11-16.

(84) Reveille JD, Durban E, MacLeod-St.Clair MJ, Goldstein R, Moreda R, Altman RD et al. Association of amino acid sequences in the HLA-DQB1 first domain with the antitopoisomerase 1 autoantibody response in scleroderma (progressive systemic sclerosis). J Clin Invest 1992; 90:973-980.

(85) Kuwana M, Kaburaki J, Okano Y, Inoko H, Tsuji K. The HLA-DR and DQ genes control the autoimmune response to DNA topoisomerase I in systemic sclerosis (scleroderma). J Clin Invest 1993; 92:1296-1301.

(86) Kuwana M, Okano Y, Kaburaki J, Inoko H. HLA class II genes associated with anticentromere antibody in Japanese patients with systemic sclerosis (scleroderma). Ann Rheum Dis 1995; 54:983-987.

(87) Salamon H, Klitz W, Easteal S, Gao X, Erlich HA, Fernandez-Vina M et al. Evolution of HLA class II molecules: alleleic and amino acid site variability across populations. Genetics 1999; 152:393-400.

(88) Masi AT, Feigenbaum RT, Chatterton RT, Cutolo M. Integrated hormonal-immunological-vascular (H-I-V triad) systems interactions in the rheumatic diseases. Clin Exp Rheumatol 1995; 13:203-216.

(89) Cutolo M, Sulli A, Seriolo B, Accardo S, Masi AT. Estrogens, the immune response and autoimmunity. Clin Exp Rheumatol 1995; 13:217-226.

(90) Nelson JL. Microchimerism and the pathogenesis of systemic sclerosis. Curr Opin Rheumatol 1998; 10:564-571.

(91) Bianchi DW, Zickwolf GK, Weil GJ, Sylvester S, DeMaria MA. Male fetal progenitor cells persist in maternal blood for as long as 27 years postpartum. Proc Natl Acad Sci USA 1996; 93:705-708.

(92) Nelson JL, Furst DE, Maloney S, Gooley T, Evans PC, Smith A et al. Microchimerism and HLA-compatible relationships of pregnancy in scleroderma. Lancet 1998; 351:559-622.

(93) Artlett CM, Smith JB, Jimenez SA. Identification of fetal DNA and cells in skin lesions from women with systemic sclerosis. N Eng J Med 1998; 338:1186-1191.

(94) Bell SA, Faust H, Mittermüller J, Kolb HJ, Meurer M. Specificity of antinuclear antibodies in scleroderma-like chronic graft-versus-host disease: Clinical correlation and histocompatibility locus antigen association. Br J Dermatol 1996; 134:848-854.

(95) Seibold J. Scleroderma. In: Kelly WN, Harris ED, Ruddy S, Sledge CB, editors. Arthritis and allied conditions. Philadelphia: Saunders,W.B., 1996: 1133-1162.

(96) Nelson JL. Maternal-fetal immuology and autoimmune disease. Is some autoimmune disease auto-alloimmune or allo-autoimmune? Arthritis Rheum 1996; 39:191-194.

(97) Green MC, Sweet HO, Bunker LE. Tight skin, a new mutation of the mouse causing excessive growth of connective tissue and skeleton. Am.J.Pathol. 82, 493-512. 1976.

(98) Everett ET, Pablos JC, Harris SE, LeRoy EC, Norris JS. The tight skin (TSK) mutation is closely linked to B2M on mouse chromosome 2. Mammalian Genome 1994; 5:55-57.

(99) Siracusa LD, Christner P, Mcgrath R, Mowers SD, Nelson KK, Jimenez SA. The tight skin (Tsk) mutation in the mouse, a model for human fibrotic diseases, is tightly linked to the beta 2-microglobulin (B2m) gene on chromosome 2. Genomics 1993; 17:748-751.

(100) Siracusa LD, Mcgrath R, Ma Q, Moskow JJ, Manne J, Christner PJ et al. A tandem duplcation within the fibrillin 1 gene is associated with the mouse tight skin mutation. Genome Research 1996; 6:300-313.

(101) Christner PJ, Peters J, Hawkins D, Siracusa LD, Jiménez SA. The tight skin 2 mouse. An animal model of scleroderma displaying cutaneous fibrosis and mononuclear cell infiltration. Arthritis Rheum 1995; 38:1791-1798.

(102) Christner PJ, Siracusa LD, Hawkins DF, Mcgrath R, Betz JK, Peters J et al. A high resolution linkage map of the tight skin 2 (Tsk2) locus: a mouse model for scleroderma (SSc) and other cutaneous fibrotic diseases. Arthritis Rheum 39, 1234. 1996.

(103) Gershwin ME, Abplanalp H, Castles JJ, Ikeda RM, Van Der Water J, Eklund J et al. Characterization of a spontaneous disease of white leghorn chickens resembling progressive systemic sclerosis (scleroderma). J Exp Med 153, 1640-1659. 1981. Abstract

(104) Abplanalp H, Gershwin ME, Johnston E, Reid J. Genetic contol of avian scleroderma. Immunogenetics 1990; 31:291-295.

(105) Atkinson JP. Some thoughts on autoimmunity. Arthritis Rheum 1995; 38:301-305.

(106) Briggs DC, Welsh K, Pereira RS, Black CM. A strong association between null alleles at the C4A locus in the Major Histocompatibility Compex and systemic sclerosis. Arthritis Rheum 1986; 29:1274-1277.

(107) Briggs D, Stephens C, Vaughan R, Welsh K, Black C. A molecular and serological analysis of the major histocompatibility complex and complement component C4 in systemic sclerosis. Arthritis Rheum 1993; 36:943-957.

(108) Takeuchi F, Nabeta H, Hong GH, Matsuta K, Tokunaga K, Tanimoto K et al. C4A and C4B null alleles are genetic markers of different types of systemic sclerosis in Japanese patients. Clin Exp Rheumatol 1998; 16:55-60.

(109) Vyse TJ, Todd JA. Genetic analysis of autoimmune disease. Cell 1996; 85:311-318.

(110) Susol E, Rands AL, Herrick A, McHugh N, Barrett JH, Ollier WER et al. Association of markers for TGFβ3, TGFβ2 and TIMP1 with systemic sclerosis. Rheumatology (Formerly B J Rheum) 2000; 39:1332-1336.

(111) Susol E, Herrick A, Ollier WER, Worthington J. Candidate gene analysis in systemic sclerosis using microsatellite markers. Brit.J.Rheumatol. 37, 87. 1998.

(112) Susol E, Rands AL, Herrick A, McHugh N, Barrett JH, Ollier WER et al. Association of markers for TGFβ2, TGFβ3 and TIMP1 with systemic sclerosis. Rheumatology (Formerly B.J.Rheum.) 39, 139. 2000. Abstract.

(113) Zetterquist H, Olerup O. Identification of the HLA-DRB1*04, -DRB1*07, and -DRB1*09 alleles by PCR amplification with sequence-specific primers (PCR-SSP) in 2 hours. Human Immunol 1992; 34:64-74.

(114) Takeuchi F, Nabeta H, Frank K-H, Conrad K. Association of the TNFa13 microsatellite with systemic sclerosis in Japanese patients. Ann Rheum Dis 2000; 59:293-296.

(115) Fanning GC, Black CM, Welsh K. TNF, LTα and TNF-RII polymorphisms in systemic sclerosis. Associations with clinical subgroup, pulmonary fibrosis and raised creatinine kinase levels. Arthritis Rheum Suppl. 9, 1735. 1998. Abstract.

(116) Tan FK, John TL, Milewicz D, Arnett FC. Sequencing of hte fibrillin 1 gene in native Americans with scleroderma. Arthritis Rheum 41 (Suppl. 9), 422. 1998. Abstract.

(117) Tan FK, Stivers DN, Foster MW, Chakraborty R, Howard RF, Milewicz DM et al. Association of microsatellite markers near the fibrillin 1 gene on human chromosome 15q with scleroderma in a native American population. Arthritis Rheum 1998; 41:1729-1737.

(118) Tan FK, Stivers DN, Arnett FC, Chakraborty R, Howard R, Reveille JD. HLA Haplotypes and microsatellite polymorphisms in and around the major histocompatibility complex region in a Native American population with a high prevalence of scleroderma (systemic sclerosis). Tissue Antigens 1999; 53:74-80.

(119) Feghali CA, Wright TM. Epidemiologic and clinical study of twins with scleroderma. Arthritis Rheum 1995; 38:S308.

(120) McHugh NJ, Harvey GR, Whyte J, Dorsey JK. Segragation of autoantibodies with disease in monozygotic twin pairs discordant for systemic sclerosis: Three further cases. Arthritis Rheum 1995; 38:1845-1850.

(121) De Juan MD, Belzunegui J, Belmonte I, Barado J, Figueroa M, Cancio J et al. An immunogenetic study of familial scleroderma. Ann Rheum Dis 1994; 53:614-617.

(122) McHugh NJ, Whyte J, Artlett C, Briggs DC, Stephens CO, Olsen NJ et al. Anti-centromere antibodies in systemic sclerosis patients and their relatives: a serological and HLA study. Clin Exp Immunol 1994; 96:267-274.

(123) Tan FK, Howard RF, Reveille JD, Moulds JM, Paxton G, Arnett FC. Case control study of systemic sclerosis among Choctaw Native Americans in southeastern Oklahoma. Arthritis Rheum 37, S282. 1994. Abstract.

(124) Merriman TR, Todd JA. Genetics of autoimmune disease. Curr Opin Immunol 1995; 7:786-792.

(125) Rosenthal AK, McLaughlin JK, Linet MS, Persson I. Scleroderma and malignancy: An epidemiological study. Ann Rheum Dis 1993; 52:531-533.

(126) Abu-Shakra M, Guillemin F, Lee P. Cancer in systemic sclerosis. Arthritis Rheum 1993; 36:460-464.

(127) Higuchi M, Horiuchi T, Ishibashi N, Yoshida S, Hiho Y, Nagasawa K. Anticentromere antibody as a risk factor for cancer in patients with systemic sclerosis. Clin Rheumatol 19, 123-126. 2000.

(128) Rothfield N, Kurtzman S, Vazques-Abad D, Charron C, Daniels L, Greenberg B. Association of anti-topoisomerase I with cancer. Arthritis Rheum 1992; 35:724.

(129) Kuwana M, Fujii T, Mimori T, Kaburaki J. Enhancement of anti-DNA topoisomerase I autoantibody response after lung cancer in patients with systemic sclerosis. Arthritis Rheum 1996; 39:686-691.

(130) Haustein UF, Ziegler V. Envirnmentally Induced systemic sclerosis-like disorders. International Journal of Dermatology 1985; 24:147-151.

(131) Silman AJ, Hochberg MC. Occupational and environmental influences on scleroderma. Rheum.Dis.Clin.North Am. 22, 737-749. 1996.

(132) D'Cruz D. Autoimmune disease associated with drugs, chemicals and environmental factors. Toxicol.Lett. 112-113, 421-432. 2000.

(133) Haustein U-F, Herrmann K. Environmental scleroderma. Clinics in Dermatology 1994; 12:467-473.

(134) Steenland K, Goldsmith DF. Silica exposure and autoimmune diseases. Am J Indust Med 1995; 28:603-608.

(135) McHugh NJ, Whyte J, Harvey G, Haustein U-F. Anti-topoisomerase-1 antibodies in silica-associated systemic sclerosis. A model for autoimmunity. Arthritis Rheum 1994; 37:1198-1205.

(136) Haustein U-F, Ziegler V, Herrmann K, Mehlhorn J, Schmidt C. Silica-induced scleroderma. J Am Acad Dermatol 1989; 22:444-448.

(137) Appleton BE, Lee P. The development of systemic sclerosis (scleroderma) following augmentation mamoplasty. J Rheumatol 1993; 20:1052-1054.

(138) Spiera H, Kerr LD. Scleroderma following silicone implantation: A cumulative experience of 11 cases. J Rheumatol 1993; 20:958-961.

(139) McLaughlin J, Fraumeni J, Olsen J. RE: Breast implants, cancer and systemic sclerosis. J Natl Cancer Inst 1994; 86:1424.

(140) Burns CJ, Laing TJ, Gillespie BW, Heeringa SG, Alcser KH, Mayes MD et al. The epidemiology of scleroderma among women: Assessment of risk from exposure to silicone and silica. J Rheumatol 1996; 23:1904-1911.

(141) Frank K-H, Fussel M, Conrad K, Rihs H-P, Kock R, Gebhardt B et al. Different distribution in HLA class II and tumor necrosis factor alleles (TNF-308.2, TNFa2 microsatellite) in anti-topoisomerase I responders among scleroderma patients with and without exposue to quartz/metal dust. Arthritis Rheum 1998; 41:1306-1311.

(142) Morse JH, Fotino M, Zhang Y, Flaster ER, Peebles CL, Spiera H. Position 26 of the first domain of the HLA-DQB1 allele in post-silicone inplant scleroderma. J Rheumatol 1995; 22:1872-1875.

(143) Rein V. Sklerodermie durch trichlorathylen-einwirking? Zentralbl Arbeitsmed 1957; 7:58-60.

(144) Black CM, Welsh KI, Walker AE, Bernstein RM, Catoggio LJ, McGregor AR et al. Genetic susceptibility to scleroderma-like syndrome induced by vinyl chloride. Lancet 1983; 53-55.

(145) Nietert PJ, Sutherland SE, Silver RM, Pandey JP, Knapp.R.G, Hoel.D.G. et al. Is occupational organic exposure a risk factor for scleroderma? Arthritis Rheum 1998; 41:1111-1118.

(146) Finch WR, Rodnan GP, Buckingham RB. Bleomycin-induced scleroderma. J Rheumatol 1980; 7:651-659.

(147) Kilaru P, Kim W, Sequeira W. Cocaine and scleroderma: Is there an association? J Rheumatol 1991; 18:1753-1755.

(148) Adams LE, Mongey A-B. Role of genetic factors in drug-related autoimmunity. Lupus 1994; 3:443-447.

(149) Dunphy J, Oliver M, Rands AL, Lovell CR, McHugh NJ. Antineutrophil cytoplasmic antibodies and HLA class II alleles in minocycline-induced lupus-like syndrome. Br J Dermatol 2000; 142:461-467.

(150) Venables P, Brookes S. Retroviruses: potential aetiological agents in autoimmune rheumatic disease. Brit J Rheumatol 1992; 31:841-846.

(151) Maul GG, Jimenez SA, Riggs E, Ziemnicka-Kotula D. Determination of an epitope of the diffuse systemic sclerosis marker antigen DNA topoisomerase I: Sequence similarity with retroviral $p30^{gag}$ protein suggests a possible cause for autoimmunity in systemic sclerosis. Proc Natl Acad Sci USA 1989; 86:8492-8496.

(152) Murrell DF. A radiacal proposal for the pathogenesis of scleroderma. J Am Acad Dermatol 1993; 28:78-85.

(153) Freedman RR, Girgis R, Mayes MD. Accute effect of nitric oxide on Raynaud's phenomenon in scleroderma. Lancet 1999; 354:739-740.

(154) Casciola-Rosen L, Andrade F, Ulanet D, Wong WB, Rosen A. Cleavage by granzyme B is strongly predictive of autoantigen status: implications for initiation of autoimmunity. J Exp Med 1999; 190:815-825.

(155) Rovere P, Sabbadini MG, Fausto F, Bondanza A, Zimmermann VS, Rugarli C et al. Remnants of suicidal cells fostering systemic autoaggression: Apoptosis in the origin and maintenance of autoimmunity. Arthritis Rheum 2000; 43:1663-1672.

(156) Masi AT, Rodnan GP, Medsger TA, Jr., Altman RD, D'Angelo WA, Fries JF et al. Preliminary criteria for the classification of systemic sclerosis (scleroderma). Arthritis Rheum 1980; 23:581-590.

(157) Olerup O, Zetterquist H. HLA-DR typing by PCR amplification with sequence-specific primers (PCR-SSP) in 2 hours: An alternative to serological DR typing in clinical practice including donor-recipient matching in cadaveric transplantation. Tissue Antigens 1992; 39:225-235.

(158) Olerup O, Zetterquist H. DR "low-resolution" PCR-SSP typing - a correction and an up-date. Tissue Antigens 1994;55-56.

(159) Olerup O, Zetterquist H. HLA-DRB1*01 subtyping by allele-specific PCR amplification: A sensitive, specific and rapid technique. Tissue Antigens 1991; 37:197-204.

(160) Olerup O, Aldener A, Fogdell A. HLA-DQB1 and -DQA1 typing by PCR amplification with sequence-specific primers (PCR-SSP) in 2 hours. Tissue Antigens 1993; 41:119-134.

(161) Isenberg DA, Maddison PJ. Detection of antibodies to double stranded DNA and extractable nuclear antigen. Assoc Clin Pathol Broadsheet 1987; 117:1-8.

(162) Hoet RM, Van Venrooij WJ. B-cell epitopes of RNA autoantigens. Mol Biol Rep 1992; 16:199-205.

(163) McHugh NJ, James IE, Maddison PJ. Differential isotype recognition of two centromere associated polypeptides by immunoblotting in connective tissue disease. Clin Exp Immunol 1988; 72:457-464.

(164) Whyte J, Earnshaw WC, Champoux JJ, Parker LH, Stewart L, Hall ND et al. Detection of anti-topoisomerase I antibodies using fiull length human topoisomerase I recombinant protein purified from a baculovirus expression system. Clin Exp Immunol 1995; 100:214-218.

(165) Black CM, Welsh KI, Maddison PJ, Jayson MIV, Bernstein RM. HLA antigens, autoantibodies and clinical subsets in scleroderma. Brit J Rheumatol 1984; 23:267-271.

(166) Livingston JZ, Scott TE, Wigley FM, Anhalt GJ, Bias WB, McLean RH et al. Systemic sclerosis (scleroderma): clinical, genetic, and serologic subsets. J Rheumatol 1987; 14:512-518.

(167) Lowe KE, Norman AW. Vitamin D and psoriasis. Nutr Rev 1992; 50:138-142.

(168) Dransfield I, Cabanas C, Barrett J, Hogg N. Interaction of leukocyte integrins with ligand is necessary but not sufficient for function. J Cell Biol 1992; 116:1527-1535.

(169) Serra CR, Rodriques SH, Szajnbok FR, Silva NP, Andrade LE. Antiperinuclear factor and antibodies to the stratum corneum of rat esophagus in juvenile idiopathic arthritis. J Pediat 1999; 134:507-509.

(170) Salvarani C, Baricchi R, Macchioni P, Carbognani R, Morelli A, Lodi L et al. Anticardiolipin antibodies in Northern Italian population with PMR/GCA. Am J Med 1992; 92:712-713.

(171) Morel PA, Chang HJ, Wilson JW, Conte C, Saidman SL, Bray JD et al. Severe systemic sclerosis with anti-topoisomerase I antibodies is associated with an HLA-DRw11 allele. Hum Immunol 1994; 40:101-110.

(172) Kuwana M, Medsger TA, Jr., Wright TM. T cell proliferative response induced by DNA topoisomerase I in patients with systemic sclerosis and healthy donors. J Clin Invest 1995; 96:586-596.

(173) Harvey G, Rands AL, McHugh NJ. Anti-RNA polymerase antibodies in systemic sclerosis (SSc): association with anti-topoisomerase I antibodies and identification of autoreactive subunits of RNA polymerase II. Clin Exp Immunol 1996; 105:468-474.

(174) Payami H, Joe S, Farid NR, Stenszky V, Chan H, Yeo PPB et al. Relative predispositional effects (RPEs) of marker allelles with disease: HLA-DR and Graves disease. Am J Hum Genet 45, 541-546. 1989.

(175) Harvey G, Butts S, Rands AL, Patel Y, McHugh NJ. Clinical and serolgical associations with anti-RNA polymerase antibodies in systemic sclerosis. Clin Exp Immunol 1999; 117:395-402.

(176) Harvey GR, Butts S, Rands AL, Patel Y, McHugh NJ. Clinical and serological associations with anti-RNA polymerse antibodies in systemic sclerosis. Clin Exp Immunol 1999; 117:395-402.

(177) Takeuchi F, Nakano K, Yamada H, Hong GH, Nabeta H, Yoshida A et al. Association of HLA-DR with progressive systemic sclerosis in Japanese. J Rheumatol 1994; 21:857-863.

(178) Whyte J, Artlett C, Harvey G, Stephens CO, Welsh K, Black C et al. HLA-DQB1 associations with anti-topoisomerase-1 antibodies in patients with systemic sclerosis and their first degree relatives. J Autoimmun 1994; 7:509-520.

(179) Hoffman RW, Takeda Y, Sharp GC, Lee DR, Hill DL, Kaneoka H et al. Human T cell clones reactive against U-small nuclear ribonucleoprotein autoantigens from connective tissue disease patients and healthy individuals. J Immunol 1993; 151:6460-6469.

(180) Mohan C, Adams S, Stanik V, Datta SK. Nucleosome: A major immunogen for pathogenic autoantibody-inducing T cells of lupus. J Exp Med 1993; 177:1367-1381.

(181) Zanelli E, Gonzalez-Gay MA, David CS. Could HLA-DRB1 be the protective locus in rheumatoid arthritis. Immunol Today 1995; 16:274-278.

(182) Das P, Bradley DS, Geluk A, Griffiths MM, Luthra HS, David CS. An HLA-DRB1*0402 derived peptide (HV3 65-79) prevents collagen-induced arthritis in HLA-DQ8 transgenic mice. Hum Immunol 1999; 60:575-582.

(183) Casciola-Rosen LA, Anhalt G, Rosen A. Autoantigens targeted in systemic lupus erythematosus are clustered in two populations of surface structures on apoptotic keratinocytes. J Exp Med 1994; 179:1317-1330.

(184) Parodi A, Puiatti P, Rebora A. Serological profiles as prognostic clues for progressive systemic scleroderma: the Italian experience. Dermatologica 1991; 183:15-20.

(185) Jacobsen S, Halberg P, Ullman S, Van Venrooij WJ, Hoier-Madsen M, Wiik A et al. Clinical features and serum antinuclear antibodies in 230 Danish patients with systemic sclerosis. Br J Rheumatol 1998; 37:39-45.

(186) Arnett F, Reveille JD, Goldstein R, Pollard KM, Leaird K, Smith EA et al. Autoantibodies to fibrillarin in systemic sclerosis (scleroderma). An immunogenetic, serologic, and clinical analysis. Arthritis Rheum 1996; 39:1151-1160.

(187) Tager.R.E, Tikly M. Clinical and laboratory manifestations of systemic sclerosis (scleroderma) in Black South Africans. Brit J Rheumatol 1999; 38:397-400.

(188) Kuwana M, Inoko H, Kameda H, Nojima Y, Sato S, Nakamura K et al. Association of human leukocyte antigen class II alleles with autoantibody profiles, but not with disease susceptibility in Japanese patients with systemic sclerosis. Internal medicine 1999; 38:336-344.

(189) Satoh M, Akizuki M, Kuwana M, Mimori T, Yamagata H, Yoshida S et al. Genetic and immunological differences between Japanese patients with diffuse scleroderma and limited scleroderma. J Rheumatol 1994; 21:111-114.

(190) Kuwana M, Kaburaki J, Arnett F, Howard RF, Medsger TA, Jr., Wright TM. Influence of ethnic background on clinical and serologic features in patients with systemic sclerosis and anti-DNA topoisomerase I antibody. Arthritis Rheum 1999; 42:465-474.

(191) Cram DS, Fisicaro N, McNeilage LJ, Coppel RL, Harrison LC. Antibody specificities of Thai and Australian scleroderma sera with topoisomerase I recombinant fusion proteins. J Immunol 1993; 151:6872-6881.

(192) Greidinger EL, Flaherty KT, White B, Rosen A, Wigley FM, Wise RA. African-American race and antibodies to topoisomerase I are associated with increased severity of scleroderma lung disease. Chest 1998; 114:801-807.

(193) Okano Y, Steen VD, Medsger TA, Jr. Autoantibody reactive with RNA polymerase III in systemic sclerosis. Ann Intern Med 1993; 119:1005-1013.

(194) Vlachoyiannopoulos PG, Drosos AA, Wiik A, Moutsopoulus HM. Patients with anticentromere antibodies, clinical features, diagnosis and evolution. Brit J Rheumatol 1993; 32:297-301.

(195) Chan HL, Lee YS, Hong HS, Kuo TT. Anticentromere antibodies (ACA): clinical distribution and disease specificity. Clin Exp Dermatololgy 1994; 19:298-302.

(196) McNeilage LJ, Youngchaiyud U, Whittingham S. Racial differences in antinuclear antibody patterns and clinical manifestations of scleroderma. Arthritis Rheum 1989; 32:54-60.

(197) McHugh NJ, James IE, Kairburn K, Maddison PJ. Autoantibodies to mitochondrial and centromere antigens in primary biliary cirrhosis and systemic sclerosis. Clin Exp Immunol 1990; 81:244-249.

(198) Zimmermann C, Steiner G, Skriner K, Hassfeld W, Petera P, Smolen JS. The concurrence of rheumatoid arthritis and limited systemic sclerosis. Arthritis Rheum 1998; 41:1938-1945.

(199) Misra R, Darton K, Jewkes RF, Black CM, Maini RN. Arthritis in scleroderma. Brit J Rheumatol 1995; 34:831-837.

(200) Nakano M, Ohuchi Y, Hasegawa H, Kuroda T, Ito S, Gejyo F. Clinical significance of anticentromere antibodies in patients with systemic lupus erythematosus. J Rheumatol 2000; 27:1403-1407.

(201) Caramaschi P, Biasi D, Carletto A, Manzo T, Randon M, Zeminian S et al. Sjogren's syndrome with anticentromere antibodies. Rev Rheum Engl Ed 1997; 64:785-788.

(202) Reveille JD, Owerbach D, Goldstein R, Moreda R, Isern RA, Arnett FC. Association of polar amino acids at position 26 of the HLA-DQB1 first domain with the anticentromere autoantibody response in systemic sclerosis (scleroderma). J Clin Invest 1992; 89:1208-1213.

(203) Earnshaw WC, Sullivan KF, Machlin PS, Cooke CA, Kaiser DA, Pollard TD et al. Molecular cloning of cDNA for CENP-B, the major human centromere autoantigen. J Cell Biol 1987; 104:817-829.

(204) Sugimoto K, Kuriyama K, Himeno M, Muro Y. Epitope mapping of human centromere autoantigen centromere protein C (CENP C); Heterogeneity of anti-CENP-C response in rheumatic disease. J Rheumatol 1998; 25:474-481.

(205) Rattner JB, Rees J, Arnett FC, Reveille JD, Goldstein R, Fritzler MJ. The centromere kinesin-like protein, CENP-E. An autoantigen in systemic sclerosis. Arthritis Rheum 1996; 39:1355-1361.

(206) Vazques-Abad D, Grodzicky T, Senecal J-L. Anticentromere autoantibodies in patients without Raynaud's disease or systemic sclerosis. Clinical Immunology 1999; 90:182-189.

(207) Russo K, Hoch S, Dima C, Varga J, Teodorescu M. Circulating anticentromere CENP-A and CENP-B antibodies in patients with diffuse and limited systemic sclerosis, systemic lupus erythematosus, and rheumatoid arthritis. J Rheumatol 2000; 27:142-148.

(208) Ferri C, Bernini L, Cecchetti R, Latorraca A, Marotta G, Pasero G et al. Cutaneous and serologic subsets of systemic sclerosis. J Rheumatol 1991; 18:1826-1832.

(209) Tubach F, Hayem G, Elias A, Nicaise P, Haim T, Kahn MF et al. Anticentromere antibodies in rheumatologic practice are not consistntly associated with scleroderma. Rev Rheum Engl Ed 1997; 64:362-367.

(210) Migliaresi S, Picillo U, Tirri G, Improta FDG, Palombara FLa, Giordano M. Infrequency of anticentromere antibody in patients without systemic sclerosis and without Raynaud's phenomenon. Arthritis Rheum 1987; 30:358-359.

(211) Picillo U, Migliaresi S, Marcialis M, Ferruzzi AM, Tirri G. Clinical setting of patients with systemic sclerosis by serum autoantibodies. Clinical Rheumatology 1997; 16:378-383.

(212) Kallenberg CGM, Pastoor GW, Wouda AA, The TH. Antinuclear antibodies in patients with Raynaud's phenomenon: clinical significance of anticentromere antibodies. Ann Rheum Dis 1982; 41:382-387.

(213) Mitchell SR, McCarty GA, Katz P. Anticentromere antibody is not specific for connective tissue diseases with promising prognosis. Arthritis Rheum 1985; 28:1076-1077.

(214) Horiki T, Moriuchi J, Takaya M, Uchiyama M, Hoshina Y, Inada K et al. The coexistence of systemic sclerosis and rheumatoid arthritis in five patients. Clinical and immunogenetic features suggest a distinct entity. Arthritis Rheum 1996; 39:152-156.

(215) Jimenez SA, Hitraya E, Varga J. Pathogenesis of scleroderma. Rheum Dis Clin North Am 1996; 22:647-674.

(216) Roberts A, Sporn M, Assoian R. Transforming growth factor β: Rapid induction of fibrosis and angiogenesis in vivo and collagen formation in vitro. Proc Natl Acad Sci U S A 1986; 83:4167.

(217) Keystone EC, Lok C, Appleton B. Elevated serum levels of transforming growth factor β in patients with scleroderma (SSc). Arthritis Rheum 35, S206. 1992. Abstract.

(218) Querfeld C, Eckes B, Huerkamp C, Krieg T, Sollbergs J. Expression of TGFβ1, TGFβ2 and TGFβ3 i localised and systemic sclerosis. Journal of dermatological Science 1999; 21:13-22.

(219) Border WA, Ruoslahti E. Transforming growth factor-β in disease: the dark side of tissue repair. J Clin Invest 1992; 90:1-7.

(220) Forberg K, Valyi-Nagy I, Heldin C-H, Herlyn M, Westmark B. Platelet derived growth factor (PDGF) in oncogenesis: development of a vascular connective tissue stroma in xenotransplanted human melanoma producing PDGF-BB. Proc Natl Acad Sci U S A 1993; 90:393-397.

(221) Takemura H, Suzuki H, Fujisawa H, Yuhara T, Akama T, Yamane K et al. Enhanced interleukin 6 production by cultured fibroblasts from patients with SSc in response to platelet derived growth factor. J Rheumatol 1998; 25:1534-1539.

(222) Kikuchi K, Kadono T, Furue M, Tamaki K. Tissue inhibitor of metalloproteinase 1 (TIMP-1) may be an autocrine growth factor in scleroderma fibroblasts. J Invest Dermatol 1997; 108:281-284.

(223) Andikopoulos K, Liu X, Kene DR, Jaenisch R, Ramirez F. Targeted mutation in the col5a2 gene reveals a regulatory role for type V collagen during matrix assembly. Nature Genet 1995; 9:31-36.

(224) Majewski S, Hunzelmann N, Schirren CG, Mauch C, Aumailley M, Krieg T. Increased adhesion of fibroblasts from patients with scleroderma to extracellular matrix components: In vitro modulation by IFN-gamma but not by TGF-β. J Invest Dermatol 1992; 98:86-91.

(225) Ford AL, Kurien BT, Harley JB, Scofield RH. Anti-centromere autoantibody in a patient evolving from a lupus/sjogren's overlap to the CREST varient of scleroderma. J Rheumatol 1998; 25:1419-1424.

(226) Awad MR, El-Gamel A, Hasleton P, Turner DM, Sinnott PJ, Hutchinson IV. Genotypic variation in the transforming growth factor-beta 1 gene: association with transforming growth factor beta 1 production, fibrotic lung disease, and graft fibrosis after lung transplantation. Transplantation 1998; 66:1014-1020.

(227) O'Kane S, Ferguson MW. Transforming growth factor betas and wound healing. Int J Biochem Cell Biol 1997; 29:63-78.

(228) Shah M, Forman DM, Ferguson MW. Neutralisatation of TGF-beta 1 and TGF-beta 2 or exogenous addition of TGF-beta 3to cutaneous rat wounds reduces scarring. J Cell Sci 1995; 108:985-1002.

(229) Gilchrist FC, Bunn C, Foley PJ, Black CM, Welsh KI, du Bois RM. Class II HLA associations with autoantibodies in scleroderma: a highly significant role for HLA DP. Genes Immunology 2001; 2:76-81. 2001.

(230) HLA 1991. Proceedings of the 11th International histocompatibility Workshop and conference. Oxford University Press 1992. Ed: Tsuji. K., Aizawa. M., Sasazuki. T. pg1166-1168.